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DIFFERENTIATION OF ENTERIC BACTERIA BY  
PYROLYSIS-GAS-LIQUID CHROMATOGRAPHY

by

Douglas J. Hanson

B.A., Macalester College, 1972

Presented in partial fulfillment of the requirements  
for the degree of

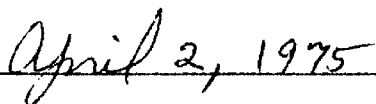
Master of Science

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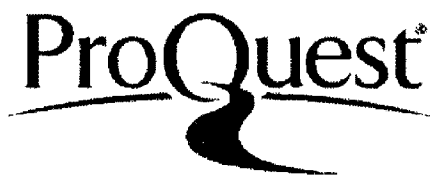


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Microbiology

Differentiation of Enteric Bacteria by Pyrolysis-Gas-Liquid Chromatography (78 pp.)

Director: Jon A. Rudbach



Previous research had shown that pyrolysis coupled with gas-liquid chromatography (PGLC) could be used to differentiate species within selected groups of microorganisms. Specific genera examined were several Penicillium and Clostridium species. In these studies, it was shown that PGLC could make gross distinctions at the species level, but it lacked resolution at the strain level.

The present study was undertaken in an attempt to extend PGLC resolution to more closely related organisms and to develop techniques for analyzing data from pyrolysis of microorganisms. In order to quantify the results of PGLC, a defined system of bacterial materials derived from Gram-negative enteric bacteria was employed. These materials included native protoplasmic polysaccharide (NPP), lipopolysaccharide (LPS), crude cell extracts of LPS, and whole cells from Escherichia coli 0113 and E. coli 0111:B4. In addition, LPS and whole cells from Salmonella species were included. Because the chemical and physical structure and antigenic relationships between these materials were known, it was possible to interpret the resolution of PGLC in terms of the structures of the materials being examined.

Preliminary results indicated that resolution could be improved most effectively by optimizing conditions for both pyrolysis and gas-liquid chromatography. This was done and resolution was increased to the point to which whole cells taken directly from complex solid media could be differentiated at the strain level. Changes in pyrolysis temperature produced the most pronounced effect on resolution. In particular, it was observed that lower pyrolysis temperatures increased the number of intermediate pyrolysis products unique to the material being pyrolyzed and concurrently increased the degree of resolution obtained when whole cells were pyrolyzed.

Results from experiments on differentiation of cells taken from solid media were quantified and presented in a format which might be adaptable for computer analysis. In addition, there was a partial characterization of peaks in the pyrolysis spectrum which might be used as easily located reference peaks. These peaks could be used to index pyrolysis elution patterns for statistical analysis.

## ACKNOWLEDGEMENT

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## ABBREVIATIONS

C	Degrees centigrade
DTA	Differential thermal analysis
GLC	Gas-liquid chromatography
LPS	Lipopolysaccharide
NPP	Native protoplasmic polysaccharide
P	Pyrolysis
PEP	Pyrolysis elution pattern
PGLC	Pyrolysis coupled with gas-liquid chromatography
PSIG	Pounds per square inch (gauge)
TGA	Thermal gravimetric analysis
TSB	Trypticase soy broth

## CHAPTER I

### INTRODUCTION

#### Historical

The ability to identify bacterial species and strains rapidly and accurately is an obvious requirement for the clinical bacteriologist. It is also of considerable practical importance for the epidemiologist who deals with enteric bacilli. For example, microbiologists who work with Salmonella are confronted with over 1200 known species of this genus (6). In situations involving multiple cases of salmonellosis, identification of the strain or strains involved is essential for locating possible sources of the infection.

The earliest methods used for identification and classification of enteric bacilli consisted of tests for unique anatomical and physiological characteristics such as fermentation of specific sugars, motility, production of metabolic by-products and inhibition of growth by selective media. These tests are still used commonly, but they require several days and do not always differentiate between strains of bacteria.

Highly specific serological techniques have been developed which were based on differences in antigenic

structure. For the enteric bacilli, the antigens used were the H, O, and Vi or K (12), all of which were found on the surface of the cells. A specific antiserum to one of these antigens was prepared and the agglutination titer was quantified with a suspension of the strain to be identified. A portion of the same antisera was then adsorbed with a heterologous strain of known antigenic composition. By comparing agglutination titers before and after adsorption, it was possible to determine if the two strains were related antigenically; also, the extent of the relationship might be estimated.

Although serological techniques were capable of distinguishing organisms with minor antigenic differences and were adaptable to screening many samples over a relatively short period of time, the need of maintaining a large stock of antisera was a major disadvantage. Also, the time and complex procedures involved in identifying serologically a single sample were often extensive. As a result, new techniques which could supplement serology in the rapid identification of individual samples would be useful in large diagnostic and taxonomic laboratories.

One such technique which might be applicable was pyrolysis (P) of the bacteria coupled with gas-liquid chromatography (GLC) (16). The lexicon definition of pyrolysis is decomposition of a material by the application of, or as a result of, heat. Pyrolysis as used in analysis requires

a more restricted definition: this is the breaking or re-arranging of chemical bonds under conditions of controlled temperature.

Recent studies relating to pyrolysis have included investigations into how cellulosic materials were affected by thermal reactions (25, 26, 27, 28). The history and results of these studies on cellulosic materials are of interest in studies on the identification of microorganisms by pyrolysis coupled with gas-liquid chromatography (PGLC) because carbohydrates make up a large portion of the cell wall of many Gram-negative bacteria. The work on pyrolysis of cellulose and related materials has been reviewed in considerable detail by Shafizadeh (25).

The use of GLC to separate and quantify the products of pyrolytic decomposition was originally just one of several techniques employed for the study of pyrolytic processes. The use of pyrolysis and GLC for qualitative identification of a material was a much later development. This was a new use for pyrolysis because the end result was not elucidation of the mechanisms by which pyrolysis occurred, but rather the identification of the material being pyrolyzed.

The use of pyrolysis coupled with gas-liquid chromatography for the examination of polymeric substances found in bacteria was first attempted by Reiner (16) in 1965. He used the technique for the taxonomic evaluation of some closely related members of the bacterial genera Escherichia, Streptococcus, and Shigella. In this report, it was stated

that the organisms could be distinguished by comparing their pyrolysis elution patterns (16). In certain instances, the distinctions were minor and were based on small differences in peak areas and retention times on individual chromatograms. It was reported that all samples subjected to pyrolysis were prepared by growing the organisms in complex media, washing the cells, centrifuging, and lyophilizing the whole cell pellets. Apparently, no attempt was made to vary experimental conditions such as the temperature used for pyrolysis or the method of sample preparations. Reiner (17) continued and expanded his original studies and correlated serological studies with PGLC data. PGLC was performed on coded samples of known serotypes and the pyrograms were then classified according to the relationship of certain specific peaks. It was postulated that this method of classification would correspond with known serological relationships (17). However, this was not demonstrated since the PGLC groups could not be correlated with the immunological reactions. Additional data was accumulated (17) during the course of these experiments which showed that pyrolysis profiles from the same organism, isolated from different human sources, were identical. Also, it was demonstrated that the medium upon which the cells were cultured seemed to have little effect on the pyrograms. Again, differentiation between closely related bacterial strains was based on minor differences in the GLC elution patterns of pyrolysis products.

Cone and Lechowich (7) extended the use of PGLC to studies on various types and strains of Clostridium botulinum. They obtained pyrolysis profiles on spores, vegetative cells, and toxin in an effort to determine the usefulness of PGLC in identifying the type of toxin causing cases of botulism. These results confirmed that PGLC had a definite advantage over the standard methods currently in use in that the time required for assays of C. botulinum was reduced from five days to between two and three days. However, considerable difficulty was experienced in identifying different strains of the same toxigenic type. Also, these investigators did not report any attempt to vary the pyrolysis temperature or other conditions such as sample preparation, age of cultures, or culture media; they followed very closely previously reported procedures (7).

Vincent and Kulik (13) also demonstrated the general capabilities of PGLC for differentiating closely related microorganisms in the course of studies on some common spoilage fungi. In these investigations, techniques were introduced for dealing quantitatively with PGLC data. A modified form of the method of Sokal and Sneath (33) was used to express percentages of similarity between all possible combinations of the 27 strains of Penicillium sp. examined. The percentage of similarity was equal to the number of similar peaks found in the pyrograms of two strains, divided by the total number of peaks found in that member



of the pair having the smallest number of peaks. Vincent and Kulik (13) also suggested that the data could be adapted to a three dimensional presentation suitable for computer analysis. Another important point brought out in this study was that strain differences could be shown by quantitative variations in the peaks and not solely by the presence or absence of characteristic peaks.

Up to this time, little effort had been devoted to research on the nature of the pyrolytic reactions and their possible effect on the resolution of pyrolysis as it related to microorganisms. This type of study was now required in the search for a means of overcoming the lack of resolution inherent in earlier investigations. In addition, it would be beneficial also, in terms of time and effort, if pyrolysis could be performed successfully on fresh whole cells rather than on lyophilized preparations or extracts. For PGLC to show significant advantages over conventional methods of bacterial identification, it was necessary that the increased resolution and decreased time of analysis inherent in PGLC be optimized.

In order to study further the characteristics of the PGLC assay of bacteria under carefully controlled conditions, first a system of chemically defined extracts had to be studied before more complicated analyses on whole cells could be performed. Because of the extensive information on the chemotypes of O-antigens in a number of enteric bacilli (10,11), and because of certain chemical considera-

tions which will be discussed later, extracts containing the O-antigen were thought to be useful materials for evaluating the effectiveness of PGLC in differentiating the enteric bacteria. A model system containing the O-antigen was available for these studies in the form of lipopolysaccharide (LPS) and protoplasmic polysaccharide (NPP) which could be obtained from Escherichia coli.

LPS, a component of the cell wall of Gram-negative bacteria(5), could be extracted in a relatively pure form by a variety of methods (4, 15, 35). This extracted material has been shown to consist of an O-specific sugar chain characteristic of the bacterial species from which it was extracted, a basal core and a lipid A segment (11). A naturally occurring material could also be extracted with trichloroacetic acid from the protoplasmic fractions of certain strains of E. coli (1, 22, 23, 34). This material was called native protoplasmic polysaccharide. It was related antigenically to LPS from the same bacterial species (1), but it did not possess the biological activities associated with LPS (22). This protoplasmic material appeared to be composed of polysaccharide chains having the same antigenic specificity as endotoxin derived from the homologous source.

The usefulness of this system lay in the fact that the O-somantic antigens used for identification of Gram-negative enteric bacteria were found in the LPS (10, 11). By

studying pyrograms of the O-specific sugar chains found in NPP and then pyrograms of the entire LPS extract, the resolution of the PGLC technique could be compared directly with known differences in immunochemical structure. Use of this system would also allow correlations to be made between PGLC elution patterns and the presence or absence of certain chemical moieties. For example, a determination could be made as to the significance of the lipid A and basal core regions of LPS for resolution of PGLC elution patterns of LPS. This could be done by pyrolyzing NPP (which lacks the basal core and lipid A segments) and comparing the resolution of PGLC elution patterns from LPS and NPP when differentiating closely related strains. The same type of comparison could be made between LPS and whole cells.

As a means of establishing further the chemical relationship between bacterial materials and pyrolysis elution peaks, it was hoped that basic qualitative analysis of the composition of individual peaks could be undertaken. Simmonds (30) reported an extensive analysis of the individual pyrolysis elution pattern peaks of Micrococcus luteus and Bacillus subtilis var. niger by combining PGLC and mass spectroscopy of the effluent. Simmonds (30) determined the chemical identity of each pyrolysis peak and showed that it was possible to identify the chemical origin (protein, carbohydrate, nucleic acid, lipid, or porphyrin) of the

materials being pyrolyzed. This was accomplished by comparing results of pyrolysis of M. luteus and B. subtilis var. niger preparations with the results of previous work (29, 31) showing that the major classes of biological materials were each degraded to characteristic pyrolysis fragments. Although this work was designed to show that PGLC and mass spectroscopy could distinguish materials of microbial origin from other organic materials in soil, during the course of these studies it was confirmed that there were unique pyrolysis products which could be attributed to each of the organisms tested. This work was the first direct evidence that there were pyrolysis products which could be related to distinct differences in the chemical structure of microorganisms.

#### Statement of Thesis

The objective of this current research was to use information gained from previous pyrolysis research which could be applied to identification of microorganisms with PGLC. This information would include material relating to variations on the conditions of pyrolysis as well as studies on PGLC identification of microorganisms. It was felt that this approach would provide a theoretical basis for modifications in PGLC techniques which might result in improved resolution. An additional objective of this project was to find a more refined means for dealing quantitatively with the collected data. Various authors have reported methods

for expressing results of GLC and PGLC including analysis of peak height and width (2) and expressions of similarity between pyrolysis elution patterns of closely related strains (13). The most common technique was a subjective comparison of the patterns (16). It was felt however, that the better resolution which was achieved provided the opportunity for adapting techniques to deal mathematically with peak heights and widths, and to achieve a more precise analysis of similarities and differences between the pyrograms and thus the bacterial strains.

With the above objectives established, the experimental design was set up as follows: 1. Preliminary experiments were carried out to establish the resolution of PGLC when separating purified extracts from the cell wall of Gram-negative bacteria; 2. Further experiments were undertaken to test the resolution of PGLC when applied to more complex materials such as whole cells grown in complex media; 3. Variations were made in the method of sample preparation time; 4. These studies were correlated with previous work which related to the mechanisms of pyrolysis and which suggested changes in experimental design leading to improved resolution; 5. Methods were suggested for the quantitative evaluation of pyrograms; 6. Finally, it was the ultimate goal of this research to develop PGLC identification of enteric bacilli to the point where it would provide a useful alternative to classical techniques in a wide variety of clinical and taxonomic applications.

## CHAPTER II

### MATERIALS AND METHODS

#### Pyrolysis and Gas-Liquid Chromatography

The method chosen for separation of the pyrolysis products was conventional gas-liquid chromatography. The gas chromatograph used was a Packard model 409 fitted with flame ionization detectors and having provisions for programmed temperature control of the column temperature.

The columns were prepared from two eight foot lengths of stainless steel tubing (Applied Science Laboratories Inc.), having an inside diameter of 0.085 inch. A calcine-fluxed diatomaceous earth, 100-120 mesh Gas Chrom Q (Applied Science Laboratories Inc.), was used as the solid support.

The stationary phase, 20 M carbowax (Applied Science Laboratories Inc.) was dissolved in chloroform, mixed with the Gas-Chrom Q in a 10% by weight ratio of carbowax to Gas-Chrom Q, and the chloroform allowed to evaporate. The coated support was then packed into the columns. The 20 M carbowax was chosen as the stationary phase because this non-polar material had been found to separate effectively the acids, aldehydes and ketones which are the predominant components of pyrolysis effluent (24). Also, high molecular weight carbowax had a small number of end groups which

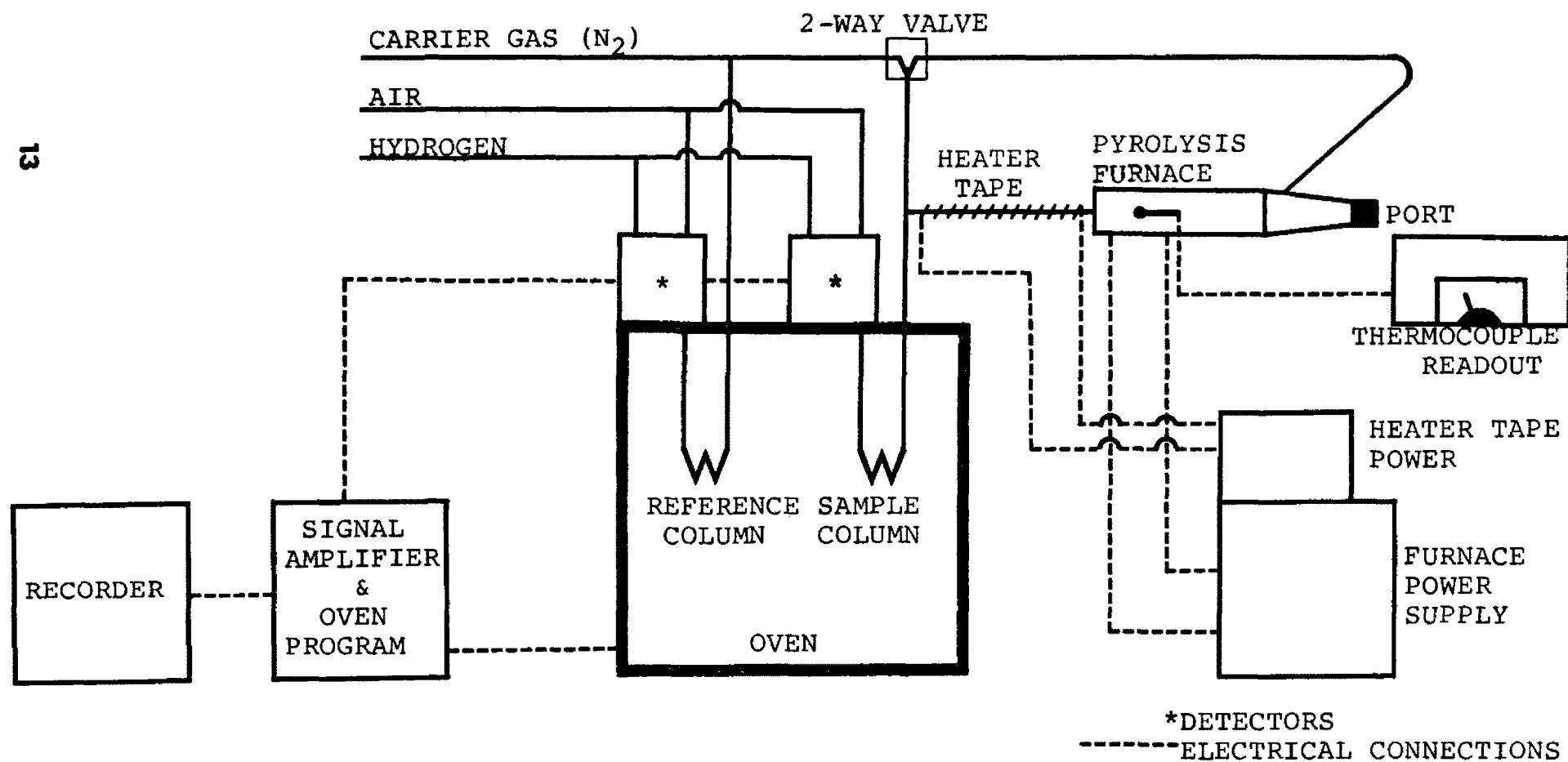
further decreased the polarity of carbowax and increased its stability during high temperature elution programs.

The pyrolysis equipment used for this study was patterned after equipment used in the Wood Products Lab at the University of Montana. The power source for the pyrolysis furnace was an F & M Scientific Pyrolysis Unit (F & M Scientific Model 80), modified to deliver a pre-determined amount of current to a furnace consisting of furnace wire wrapped around a Pyrex tube (see Figure 1). The furnace was encased in asbestos and incorporated a chromel-alumel thermocouple for precise measurement of oven temperature.

The design of the furnace was such that pyrolysis occurred in a nitrogen atmosphere and this same nitrogen was then used to sweep the pyrolysis products into the chromatograph column. To accomplish the movement of the effluent from the furnace into the chromatograph, a teflon tube was attached to the outlet of the furnace and a syringe needle suitable for insertion into the septum of the gas chromatograph was attached to the other end of the tube. This tube was enclosed in heater tape to keep the teflon tube at approximately 300 C. This was done to prevent less volatile pyrolysis products from condensing on the walls of the teflon tube.

Originally the carrier gas was allowed to flow through the furnace throughout the development of the pyrolysis

Fig. 1. Schematic diagram of pyrolysis and gas-liquid chromatography apparatus. Valve is used to shunt carrier gas through pyrolysis furnace during 5 minutes following placement of sample in furnace. Carrier gas then flows directly into sample column.





elution pattern. This procedure was later changed because effluent which had condensed on the walls of the pyrolysis furnace was continually bleeding into the columns and thus caused a shift in the recorder baseline. A valving system was constructed at the carrier gas inlet to the gas chromatograph so that the carrier gas was channeled into the pyrolysis furnace only during the five minutes immediately following the introduction of the sample into the pyrolysis furnace. The carrier gas was then allowed to flow directly into the column and the inlet end of the pyrolysis furnace was closed off. A considerable reduction in baseline drift was noted after this procedure was initiated, and it was then possible to use less attenuation and thus much smaller samples could be employed.

Introduction of the samples into the pyrolysis furnace was accomplished by placing the desired amount of material to be pyrolyzed (usually between 500 ug and 1 mg) into a small aluminum combustion boat, and then placing the boat into the cool end of the pyrolysis furnace through a sealable port. A small teflon coated magnet was then placed into the furnace behind the boat and the port was closed. The furnace was purged with carrier gas to remove residual air and the current to the pyrolysis furnace was adjusted to give the desired operating temperature. The sample was pyrolyzed by sliding the aluminum boat into the furnace using magnetic coupling between the magnet previously placed in the

furnace and another magnet being manipulated along the outside wall of the glass furnace tube. With this technique, the time of introduction of the sample into the furnace could be controlled precisely in relation to the furnace temperature, and there was no contamination of the furnace by atmospheric air during introduction of the sample.

The advantages of this method of pyrolysis over the ribbon method of pyrolysis (flash pyrolysis) used by previous workers were that relatively large quantities of material (up to 3 mg) could be used and the amount of material could be controlled precisely by weighing the pyrolysis boats and material to be analyzed. Also, a large measure of control was afforded to the method by the incorporation of a thermocouple directly into the furnace. Temperatures in this particular furnace could be varied from 250 C to 600 C, allowing close examination of the effects of temperature on the pyrolysis elution patterns.

The chromatograph was fitted with two identical columns and two identical flame ionization detectors. The column detectors were operated with their outputs connected in series but having opposite polarities. This was done so that any common mode signals resulting from changes in column characteristics due to temperature programming would be canceled at the input to the signal amplifier (electrometer). Carrier gas flow rates and hydrogen and air flow rates to the detectors were balanced so that the two columns

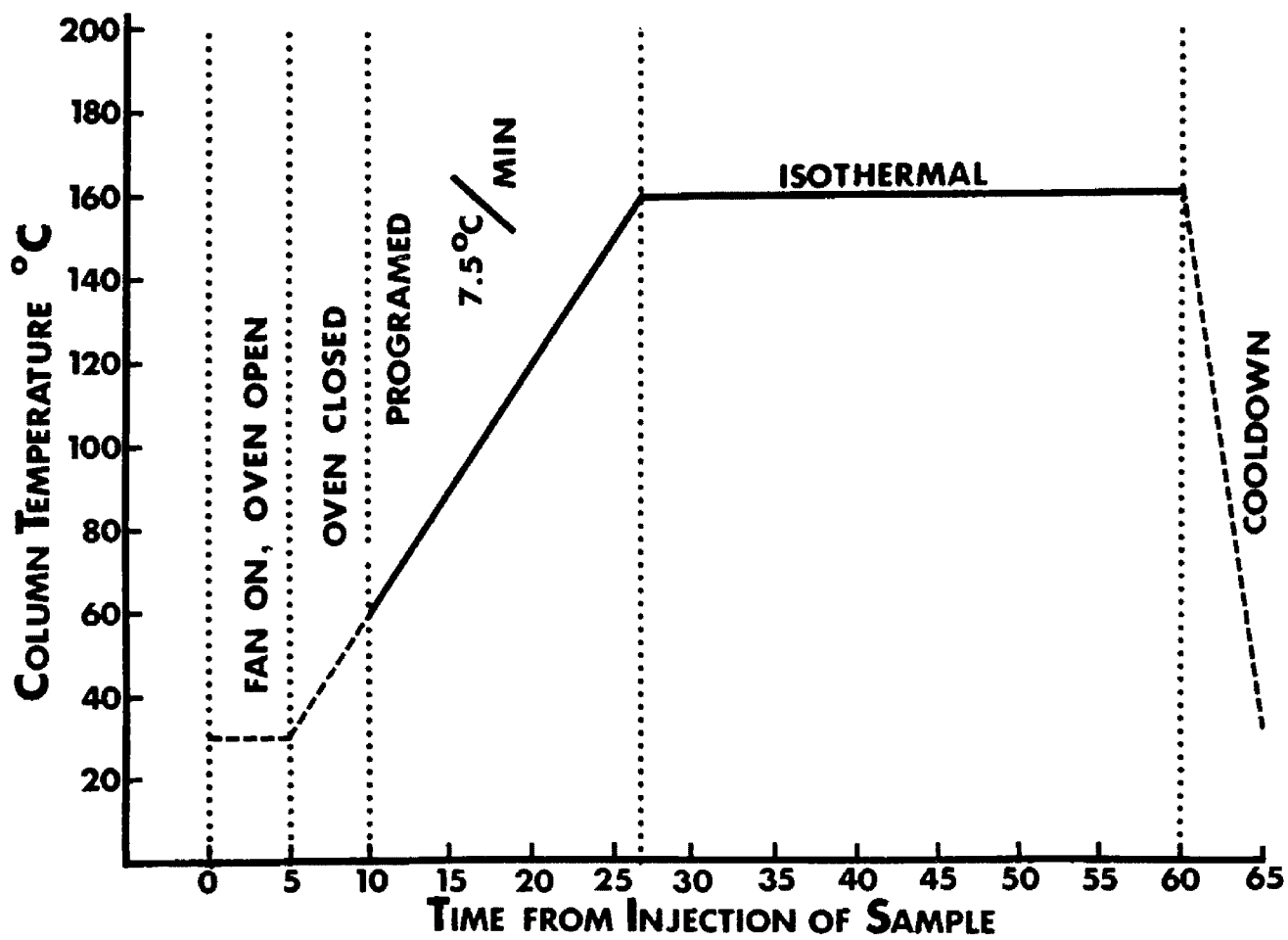
would give identical responses to identical injections of a methanol standard.

After frequent changes to improve separation, carrier gas flow rates were fixed at 28 pounds per square inch guage (psig) yielding a flow rate of 16 ml/min for the sample column and 14 psig yielding a flow rate of 5.5 ml/min for the reference column. These figures were obtained at an oven temperature of 35 C and increased with increasing oven temperature. Flow rates for the two detectors were 17 psig yielding 25 ml/min for hydrogen and 21 psig yielding 250 ml/min for air.

A considerable amount of time was devoted to determining the temperature program which gave the best resolution of the pyrolysis elution products. Initially, 180 C was used as the final column temperature, but this setting caused bleeding of the stationary phase from the column, making baseline drift compensation difficult. Best resolution was achieved by programming the column oven as shown in Figure 2 with a final oven temperature of 160 C. The injectors were maintained at 300 C and the detectors at 320 C.

Initial difficulties with baseline shift allowed a full scale deflection of no less than  $1.6 \times 10^{-9}$  amperes. Through the use of lower oven temperatures and modifications to the carrier gas pathway (as described previously), this requirement was reduced to  $2.0 \times 10^{-10}$  amperes for full

Fig. 17. Reproducibility of pyrograms from individual samples of whole cells from Salmonella typhi. Figure shows peak heights from each of 25 peak regions divided by peak height from region 1. Lines terminating with a triangle indicate that values exceeded recorder limits.



scale deflection. This output was recorded by a Honeywell Elektronik Recorder (Model 194) operating at a full scale deflection of 1 mv.

#### Analysis of NPP, LPS, and Extracted LPS

The lipopolysaccharides used in this experiment were extracted from Escherichia coli 0113 (Braude strain), Escherichia coli 0111:B4 (Difco strain), Salmonella newington, S. anatum, and S. thompson. These materials were prepared by a modification of the aqueous-phenol method of Westphal et al. (35) and were available in lyophilized form from laboratory stock (E. coli 0113 LPS; lot 181, E. coli 0111:B4 LPS, lot 178, Salmonella LPS, lot S-PP-386). The native protoplasmic polysaccharide was prepared from E. coli 0113 by trichloroacetic acid extraction of the protoplasmic fraction (34). This material was also lyophilized and available from laboratory stock (E. coli 0113 NPP, lot 98/107).

LPS and NPP samples were analyzed by determining empirically the amount of sample which afforded maximum resolution (approximately 700 µg), weighing this amount into aluminum combustion boats, pyrolyzing the sample, separating the components of the effluent on the chromatograph columns, and recording the output of the detectors.

Analysis of crude LPS extracted from whole cells of E. coli 0113 and E. coli 0111:B4 was undertaken based on a

previously demonstrable ability to identify pyrograms of purified LPS from these organisms. In the first experiment, cells were grown for 24 hrs in 10 ml of trypticase soy broth (TSB) (Baltimore Biological Laboratories lot # 106632) and washed three times with 10 ml of distilled water. The cells were resuspended in 1 ml of distilled water and boiled for 30 min. The boiled suspension was then centrifuged in a clinical centrifuge and 200  $\mu$ l of the supernatant fluid, containing the crude LPS, was placed in a tared combustion boat, dried by dessication, weighed and pyrolyzed.

#### Analysis of Whole Cells and Growth Medium

Analysis of whole cells was initiated by determining the pyrolysis elution patterns from complex medium both with and without bacterial cells. Samples of TSB were lyophilized, weighed and subjected to pyrolysis. E. coli 0111:B4 and E. coli 0113 were then cultured at 35 C in 10 ml of TSB on a shaker for 18 hr. TSB containing cells was lyophilized, pyrolyzed, and the results compared to data from the medium alone.

In order to determine whether or not peaks from the medium were masking peaks from the bacterial cells, E. coli 0113 and E. coli 0111:B4 were grown in 10 ml TSB at 35 C on a shaker. The whole cells were separated by centrifuging the cultures, washing the cells three times using 10 ml of distilled water and lyophilizing this suspension. The

lyophilized whole cells were then placed in a combustion boat, weighed, and pyrolyzed. Pyrolysis elution patterns from this experiment were compared with data from sterile medium and medium containing bacterial cells.

As a means of conserving time and materials, it was determined that drying the wet samples in a dessicator under vacuum resulted in products which gave the same pyrolysis elution patterns as those obtained by lyophilization of the samples. This observation was verified by repeating some of the previous experiments, with each wet sample placed in a tared combustion boat dessicated under vacuum for a time sufficient to dry the sample (usually five to six hours), and pyrolyzing the dried samples as before.

Results of this preliminary work suggested that the medium was contributing a substantial number of peaks to the overall pyrolysis elution pattern and it was felt that a simple chemically defined medium consisting only of essential salts and a carbon source would decrease the complexity of the chromatographs. M-9 medium (18) with 0.5% glucose was chosen as a medium suitable for growing E. coli 0111:B4 and E. coli 0113. This medium contained only glucose and essential salts. M-9 plus glucose was analyzed by itself by placing 170  $\mu$ l of the sterile medium in a combustion boat and dessicating as described previously. E. coli 0111:B4 and E. coli 0113 were then grown in 10 ml of



M-9 medium plus glucose for periods of 24 and 48 hrs. Two time periods were used to determine whether or not longer growth periods would result in a depletion of the carbon source, an increase in metabolic by-products, and, thus, a pyrolysis elution pattern more characteristic of the cell type rather than the composition of the media in which the cells were grown.

In the last portion of this work, information obtained from experiments on characterization of the pyrolysis process was applied to further research on the identification of whole cells obtained directly from the surface of complex solid media. Pyrolysis was done at 350 C and modifications to the carrier gas pathway were made as described in the section on equipment. Samples of E. coli 0113, E. coli 0111:B4, S. typhi, S. typhimurium, and S. enteriditis were grown on trypticase soy agar slants for 18 hr at 35 C. Growth on the surface of the agar was removed with a sterile loop, placed in combustion boats, and dried in a dessicator under vacuum. It was found that the amount of material required was not critical and could be estimated closely enough to eliminate the need for weighing individual samples. These combustion boats were then placed in the pyrolysis furnace without further preparation. Pyrolysis was accomplished with the same chromatograph program as was used in the previous experiments.

## Differential Thermal Analysis and Thermal Gravimetric Analysis

In order to study the temperature dependency of the pyrolysis process, thermal gravimetric analysis (TGA) and differential thermal analysis (DTA) studies were undertaken. This work was done on samples of LPS from E. coli 0113 using a Dupont Thermal Analyzer (Model #990). Data was gathered with the cooperation of Dr. Ronald A. Susott of the University of Montana Wood Products Laboratory.

DTA was accomplished by placing about 1 mg of sample in contact with a thermocouple located inside a controlled temperature furnace. The sample, its thermocouple, and a reference thermocouple were heated from room temperature to 500 C/min. The output from the two thermocouples was connected so that slight differences in temperature between the two thermocouples showed up as deviations from an otherwise horizontal baseline on the recorder. Positive peaks would indicate that the sample thermocouple had a slightly higher temperature than the reference thermocouple and thus the sample was undergoing an exothermic reaction. Negative peaks would indicate that an endothermic reaction was occurring.

Thermal gravimetric analysis was performed by heating about 1 mg of the LPS over the same temperature range and at the same rate as for DTA. The sample was placed in the weighing pan of a Cahn electrobalance (Model # R-100) before being heated in a nitrogen atmosphere and weight changes

were recorded with respect to temperature. By observing and comparing the results of DTA and TGA, it was possible to get a qualitative estimate of the nature of the pyrolysis process with respect to LPS. From this data, and from the results of previous pyrolysis experiments, it was determined that the pyrolysis temperature of all future pyrolysis experiments should be changed from 500 C to 350 C. This was done and improved distinction between pyrograms of different whole cell types was noted.

#### Qualitative Analysis of Pyrolysis Peaks

The following experiments were performed in an attempt to determine the chemical nature of selected individual peaks and to establish reference peaks which could be identified consistently. These reference peaks could be used as markers during data analysis.

The first set of experiments was performed by obtaining the less volatile components of the pyrolysis effluent in solution. By doing this, it was possible to incorporate a small quantity of a known sample into the solvent and thus look for enhancement of a peak containing both the known sample and an unknown component of the effluent. This enhancement could be taken as presumptive evidence that the known and unknown were the same because they had identical retention times. However, further chemical and physical tests would be required for absolute proof of chemical identity.

Pyrolysis products were obtained in solution by passing the effluent from pyrolysis of about 3 mg (3 - 1 mg samples pyrolyzed separately) of LPS directly into a 6 in length of 1/8 in O.D. teflon tubing submerged in an ice bath. This was accomplished by pyrolyzing the samples in the same furnace employed previously for sample pyrolysis and then allowing the effluent nitrogen to pass into the teflon tube rather than into the gas chromatograph. The volatile materials from pyrolysis of the samples were condensed on the walls of the tube and were then dissolved in methanol by passing 10  $\mu$ l of methanol back and forth through the teflon tube. Subsequently, 1  $\mu$ l of the methanol was removed with a microliter syringe (Hamilton C. Inc. model # 701) from the teflon tube and injected into the gas chromatograph. The sample was then chromatographed according to the program described previously.

The second experiment was designed to relate directly, retention times of known samples to retention times of peaks from pyrolysis products of bacterial materials. A sample of furfural was first run through the chromatograph by placing 0.1  $\mu$ l of furfural in a combustion boat, sliding this sample into the pyrolysis furnace (operating at 350 C), sweeping the effluent into the gas chromatograph, and determining the retention time with the same program as used for the previous pyrolytic analysis of bacterial materials. Furfural was used because it was sufficiently non-volatile

so that it remained in the combustion boat at room temperature without evaporating; also, it was reported to be stable at 350 C (9). Subsequently, furfural was injected directly into the gas chromatograph and the carrier gas flow rate was adjusted so that the direct injection of furfural yielded the same retention time as that for furfural evaporated in a combustion boat at 350 C. This adjustment was necessary in order to obtain a valid comparison between other more volatile known samples (which could not be retained in a combustion boat at room temperature) and products from the pyrolysis of bacterial materials. Since the carrier gas flow rate had been adjusted previously with furfural, it was hypothesized that the retention times from the other known samples would correspond to the retention times of similar materials generated during the pyrolysis of bacterial products.

The 6 knowns used in this experiment were furfural, 5-methyl furfural, 2-methyl furan, furan, acrolein, and acetaldehyde. These samples were provided by the Wood Chemistry Laboratory and were chosen because they are common materials shown by previous work (25) to arise from the pyrolysis of carbohydrates (particularly cellulose).

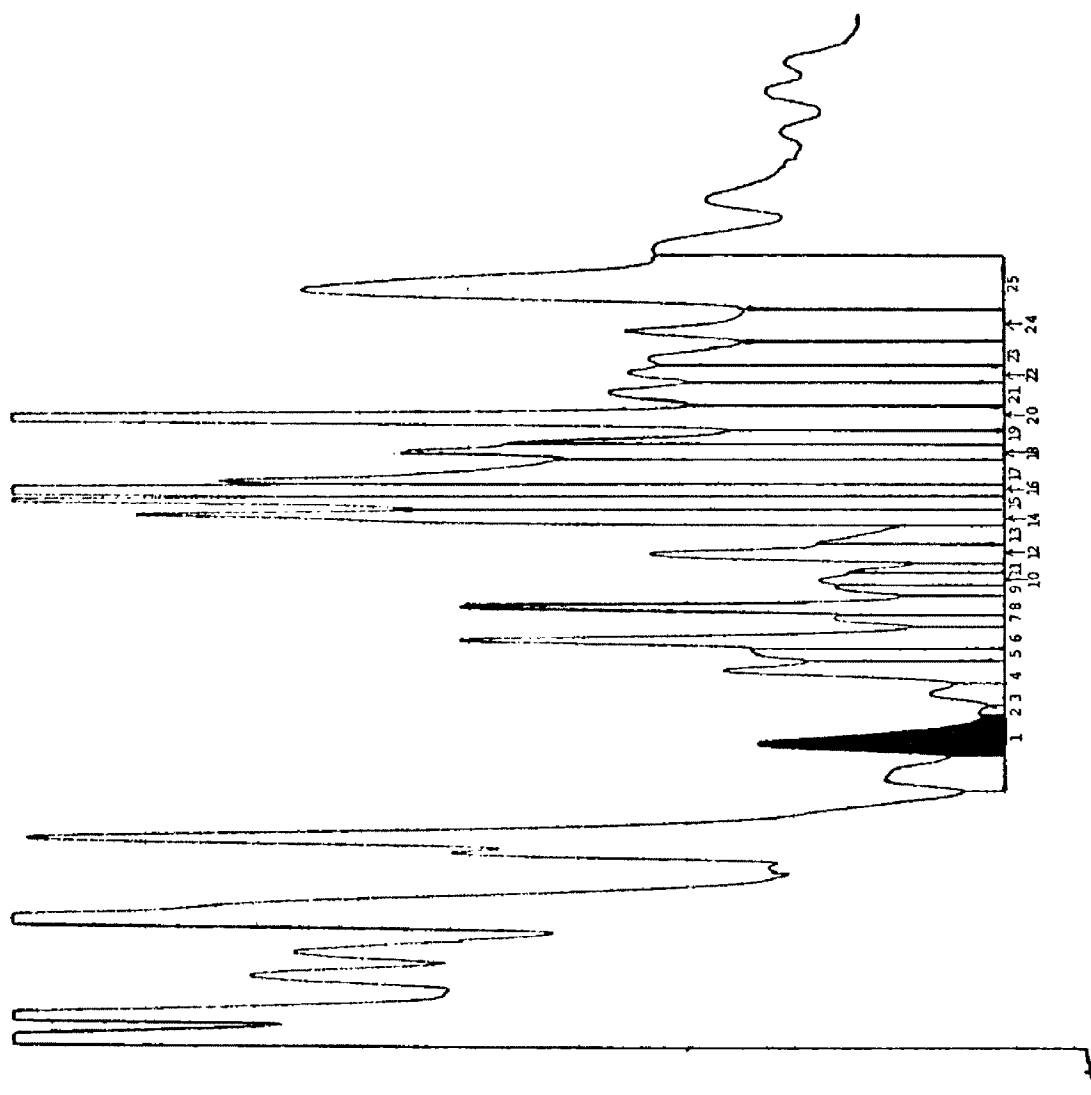
## CHAPTER III

### RESULTS

#### Analysis of NPP and LPS

The initial pyrolysis experiments were carried out in order to standardize equipment and procedures and to determine whether or not sufficient resolution could be obtained to warrant continuation of the project. Purified NPP from E. coli 0113 was chosen as the first material to be examined by PGLC for reasons discussed previously. Once reproducible pyrograms were achieved with NPP, PGLC analysis was extended to LPS and results from analysis of NPP were compared with pyrolysis elution patterns obtained from LPS from E. coli 0111:B4 and E. coli 0113. Comparison of these pyrograms and evaluation of all subsequent pyrograms were facilitated by introducing the concept of peak regions. Assignment of these peak regions was dependent on the location of a single reference peak (peak region 1) which appeared on every pyrogram. Peak region assignments are shown on a pyrogram from whole cells of S. enteriditis (Figure 3). The area of each pyrogram containing these peak regions was also marked by square brackets on all pyrograms presented here. Peak region 1 contained the reference peak mentioned previously, and this reference peak was shaded on subsequent pyrograms. These peak regions were used to set up a numerical procedure

Fig. 3. A sample pyrogram demonstrating the method for assignment of peak regions 1 through 25. Shaded area shows reference peak region which is also marked on all other pyrograms included in this section. Pyrogram in this figure was made from fresh whole cells of Salmonella enteriditis pyrolyzed at 350 C.





for analyzing data from pyrolysis elution patterns which will be described in conjunction with data from pyrolysis of fresh whole cells.

Comparison of the peak regions from NPP from E. coli 0113 with peak regions from LPS from the same organisms (Figure 4, A and B) showed that portions of the LPS, in addition to the carbohydrate, markedly contributed to the pyrolysis profile. Furthermore, these non-immunodeterminant group materials in LPS contributed to those peak regions (6 - 13) which were distinctive between pyrograms of E. coli 0113 and E. coli 0111:B4 (Figure 4, B and C). Therefore, in order to obtain maximal resolution, it was decided that a material at least as complex as LPS should be used.

From these same data it was shown that a resolution sufficient to distinguish between the pyrograms of LPS from E. coli 0113 and E. coli 0111:B4 could be obtained (note regions 6 through 13, Figure 4, B and C). As a further test for resolution, LPS from S. anatum and S. thompson were pyrolyzed and chromatographed. Pyrograms of LPS from these organisms (Figure 5) could be distinguished from one another by gross inspection. The differences between these pyrograms were noted by comparing peak heights and peak locations in regions 8 through 13 and in regions 19 through 23 marked on Figure 5. These pyrograms were also obviously different from the patterns of LPS from E. coli strains, and they could be differentiated by comparing the areas mentioned previously.

Fig. 4. Differentiation among differing preparations of bacterial polysaccharides. Pyrograms are of NPP from E. coli 0113 (A), LPS from E. coli 0113 (B), and LPS from E. coli 0111:B4 (C). Pyrograms were distinguished by comparing peak areas and locations in peak regions 6 through 13. Lyophilized material weighed 708.8 µg, 714.4 µg and 707.2 µg respectively and was pyrolyzed at 500 C.

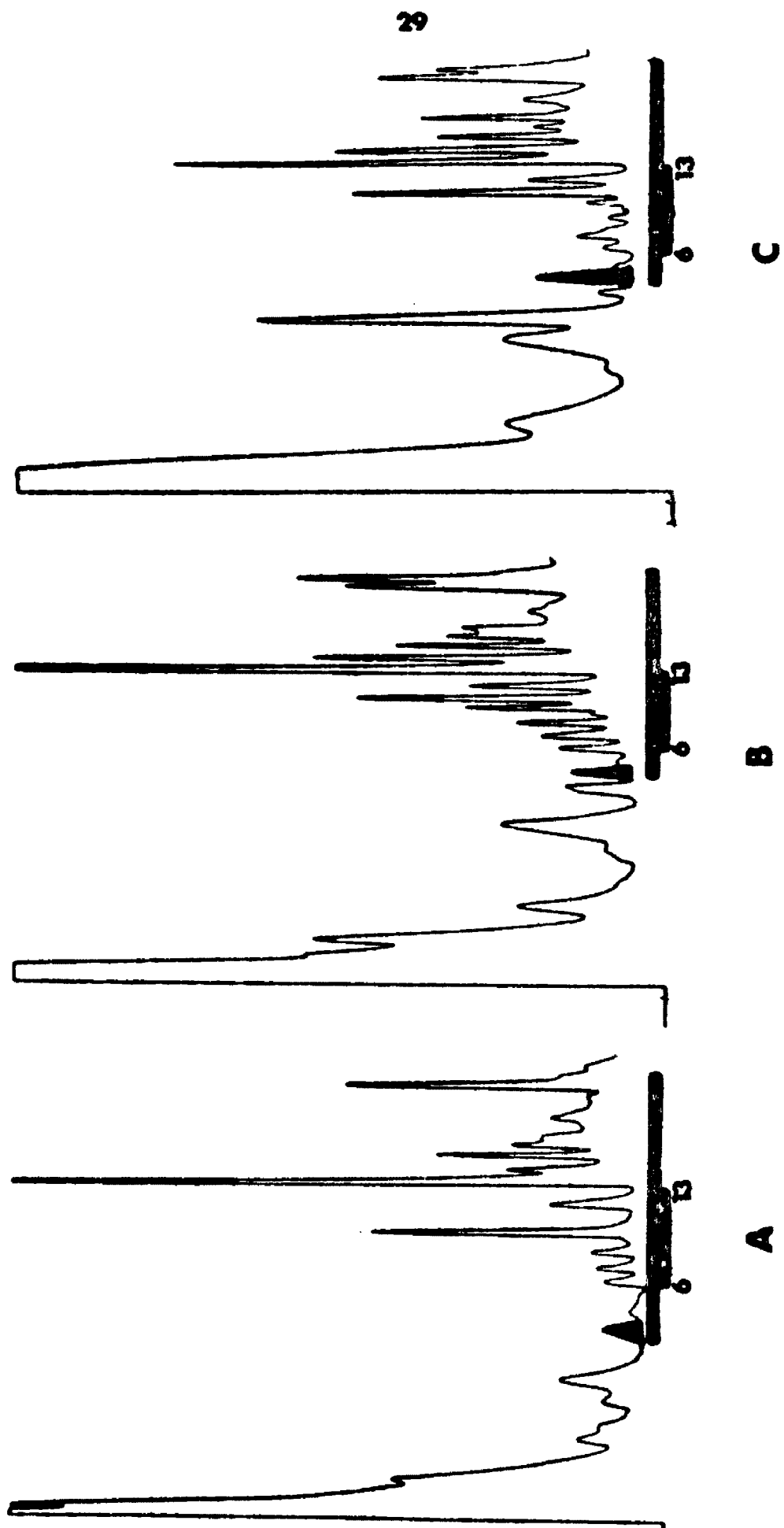
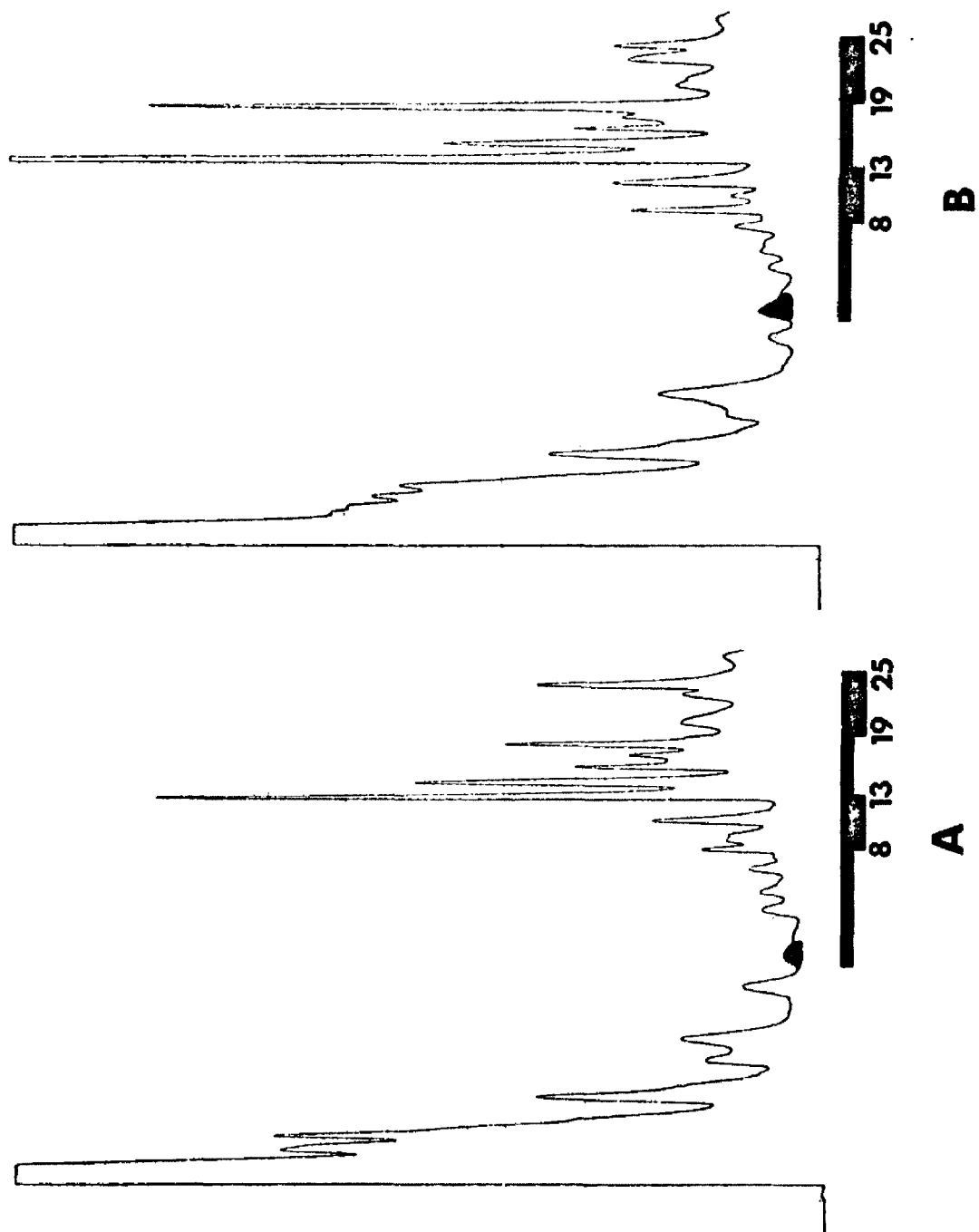


Fig. 5. A comparison of pyrolysis profiles of LPS from two species of Salmonella. Pyrograms are of LPS from Salmonella thompson (A) and LPS from Salmonella anatum (B) pyrolyzed at 500 C. Lyophilized samples weighed 712.0 µg and 712.8 µg respectively. Differentiation was accomplished by comparing peaks in regions 8 through 13 and peaks in regions 19 through 25.



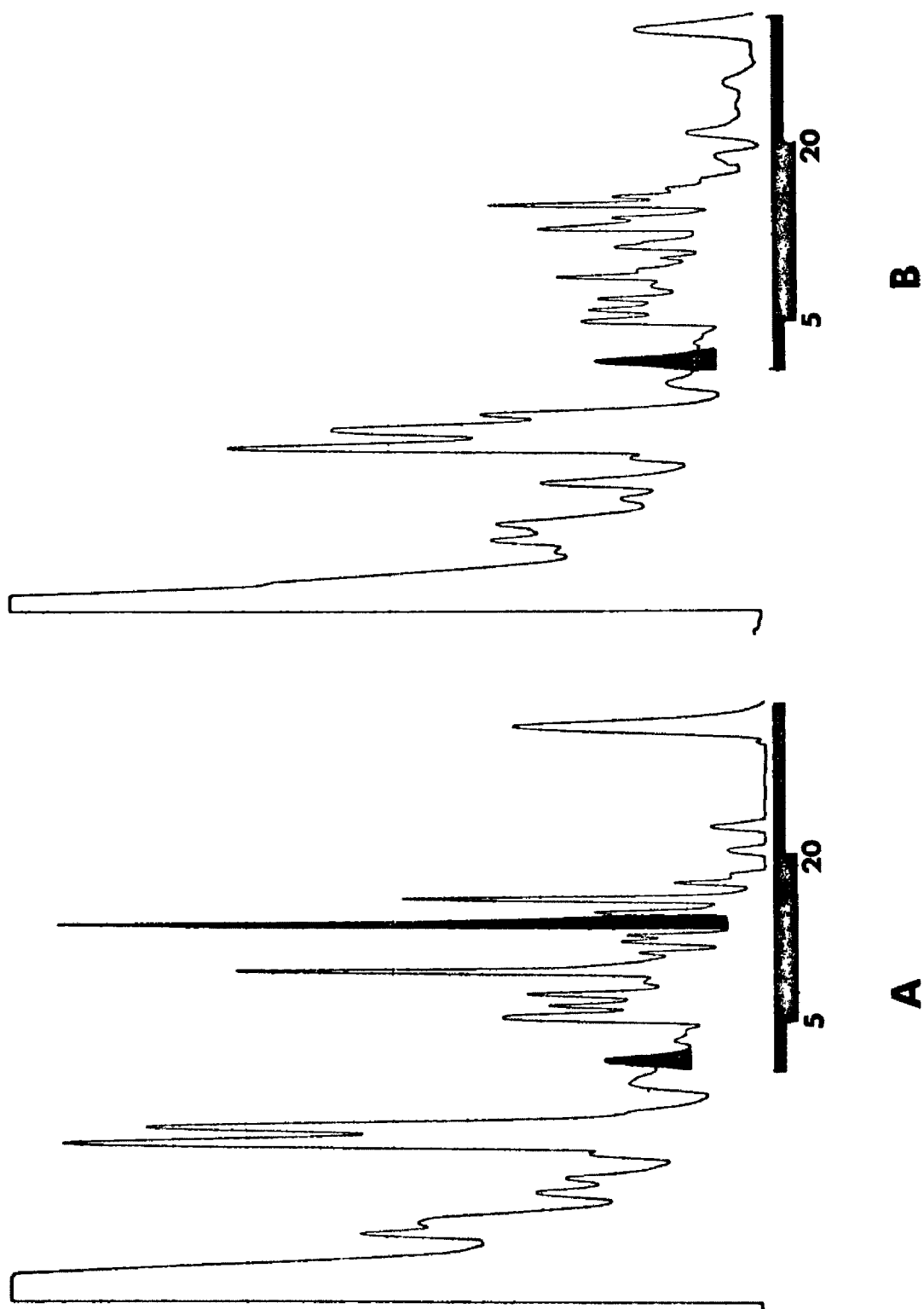
### Analysis of Crude Extracts

Because of a demonstrated ability to identify characteristic regions in purified bacterial materials, it was felt that the resolving powers of PGLC could be tested further by examining the effluent from crude extracts of whole cells. This was then a further step in the progression from pyrolysis of purified extracts to pyrolysis of whole cells taken directly from solid media.

When freshly cultured and washed whole cells from E. coli 0111:B4 were boiled for 30 min in distilled water and the supernatant fluid was placed in aluminum combustion boats, pyrolysis resulted in profiles which were unlike any previously obtained. The distinctions between pyrograms of boiled whole cells and earlier pyrograms of LPS and NPP could be made by comparing peak regions 5 through 20 (Figure 6) to these same regions on previous pyrograms. These areas could also be used to consistently differentiate between pyrograms from crude extracts of E. coli 0111:B4 and E. coli 0113. In particular, there is a distinct peak at region 15 (shaded in Figure 6, A) which does not appear in Figure 6, B).

It should be noted that peak sizes and peak locations characteristic of the various species and strains pyrolyzed were found primarily in the area defined as peak region 1 through peak region 25. Other portions of the pyrograms seemed to be quite similar from one pyrogram to the next and

Fig. 6. Pyrograms of crude LPS extracted from E. coli 0113 (A) and E. coli 0111:B4 (B). This shows how differentiation can be accomplished by comparing peaks in regions 5 through 20. Material was obtained by boiling whole cells and lyophilizing the supernatant fluid after centrifugation of the boiled cells. Pyrolysis was carried out at 500 C.





depended more on the condition of the samples when pyrolyzed (amount of dessication and method of preparation) than on the species or strain of the sample. This last observation relates particularly to that portion of the pyrogram preceding the reference peak marked on each pyrogram.

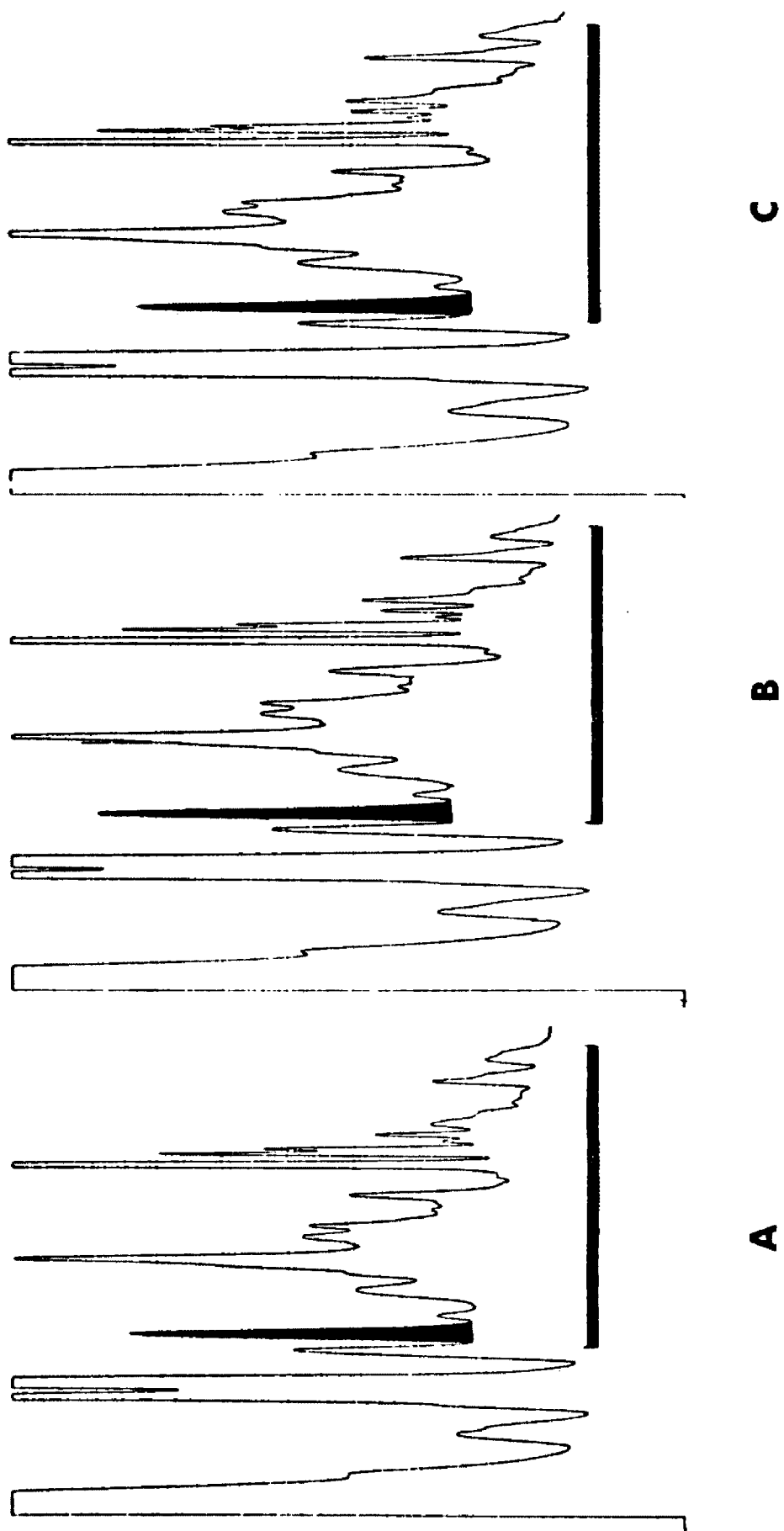
The above data provided evidence that crude extracts from fresh whole cells could be pyrolyzed so as to provide sufficient resolution to separate pyrograms from E. coli 0111:B4 and E. coli 0113. The next step was to eliminate the extraction procedure and analyze fresh whole cells taken directly from the surface of complex media. This would then decrease the amount of time required for the assay.

#### Analysis of Whole Cells in Growth Media

This series of experiments was designed to determine pyrolysis elution patterns from complex medium and chemically defined medium, both with and without bacteria. In addition, preparations of freshly cultured and washed bacterial whole cells were pyrolyzed.

Results shown in Figure 7 indicated that it was not possible to distinguish between TSB alone and TSB containing bacterial cells. It was felt that the inability to recognize the presence of cells in TSB was due to pyrolysis products of components of the broth masking entirely any distinctive features of the pyrograms contributed by the cells.

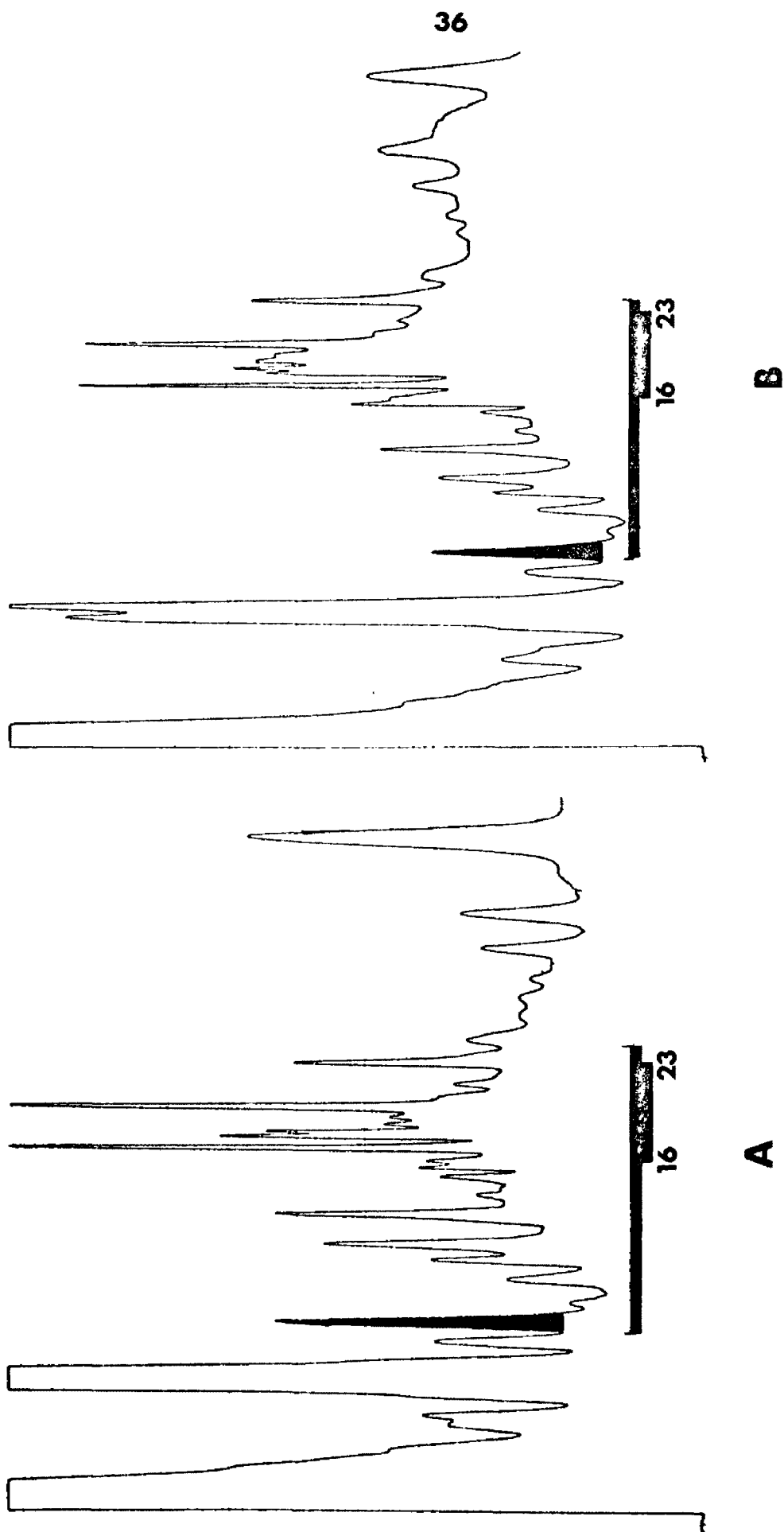
Fig. 7. Inability to differentiate profiles of cells in trypticase soy broth (TSB) from profiles of sterile broth. Pyrograms of sterile TSB (A), TSB containing whole cells of E. coli 0111:B4 (B), and TSB containing whole cells of E. coli 0113 (C). Cultures were grown for 24 hrs, 200  $\mu$ l of culture was dessicated in a combustion boat, and the samples were pyrolyzed at 500 C.



It was obvious that the materials now being analyzed were too complex to be resolved by PGLC under the present experimental conditions. The next step was to simplify the material to be analyzed to a point where resolution could be restored. This was accomplished by growing E. coli 0111:B4 and E. coli 0113 in TSB for 24 hr, washing the cells free of medium with distilled water, lyophilizing and pyrolyzing the cells. PGLC of these materials yielded profiles showing good resolution between whole cells of E. coli 0113 and E. coli 0111:B4. Pyrograms from these two organisms could be distinguished by comparing pyrogram profiles in peak regions 16 through 23 (Figure 8). However, there was little obvious correlation between pyrograms of washed whole cells of these two organisms and pyrograms of LPS from the same two organisms (compare Figure 4 and Figure 8).

Since it had now been determined that washed whole cells could be differentiated, a growth medium was sought which would allow unwashed cells to be resolved and thus eliminate the time required for washing the cells. Because the complexity of the medium was felt to be a major factor in the inability to obtain resolution from cells grown in TSB, a simple medium containing only essential salts and 0.5% glucose was examined. This medium (M-9 medium with 0.5% glucose) (18) was pyrolyzed and its program compared to those from sterile TSB. Results were obtained which

Fig. 8. Differentiation between profiles of preparations of washed whole cells. Pyrograms are of washed whole cells of E. coli 0111:B4 (A) and washed whole cells of E. coli 0113 (B). Comparison of peak regions 16 through 23 shows limited resolution available. Cells were washed from 24 hr culture in TSB, dessicated, and pyrolyzed at 500 C.



showed a profile completely dissimilar to that from sterile TSB (Figure 7, A and Figure 9, A). Figure 10, A and B showed that pyrograms of lyophilized M-9 medium containing 0.5% glucose and whole cells from 24 hr cultures of E. coli 0113 and E. coli 0111:B4 were not distinguishable from each other but were distinguishable from pyrograms of sterile M-9 + 0.5% glucose (Figure 9, A). In a further effort to obtain resolution between whole cells of E. coli 0113 and E. coli 0111:B4 pyrolyzed directly from culture in M-9 medium + glucose, additional cultures were grown for 48 hr rather than the 24 hr period previously used. Results from the 48 hr cultures are shown in Figure 9, B and C. It is important to note that the longer growth period resulted in resolution sufficient to distinguish pyrograms from cultures of the two strains of E. coli. Differentiation was made by comparing peak regions 8 through 25 (Figure 9, B and C) and also noting the presence of the peak in peak region 9 on Figure 9, C (shaded).

Results from pyrolysis of whole cells in a complex liquid medium showed that preparation of cells for analysis (i.e. extraction or washing) would be required under the present conditions of pyrolysis in order to obtain resolution sufficient to distinguish between pyrogram profiles of different bacterial strains. However, cells grown for several days in a simple medium might be distinguishable in the cultured whole cell format. Since simple media are not available for all organisms which might be subjected to PGLC

Fig. 9. Attempt to differentiate between two strains of E. coli grown in a simple defined medium for 48 hr. Included is a comparison between pyrograms of sterile M-9 + 0.5 % glucose (A), M-9 + 0.5 % glucose containing a 48 hr culture of E. coli 0113 (B), and M-9 + 0.5 % glucose containing a 48 hr culture of E. coli 0111:B4 (C). E. coli 0113 and E. coli 0111:B4 are separated by comparing peaks in peak regions 8 through 25. The shaded peak in peak region 9 is characteristic of E. coli 0111:B4.



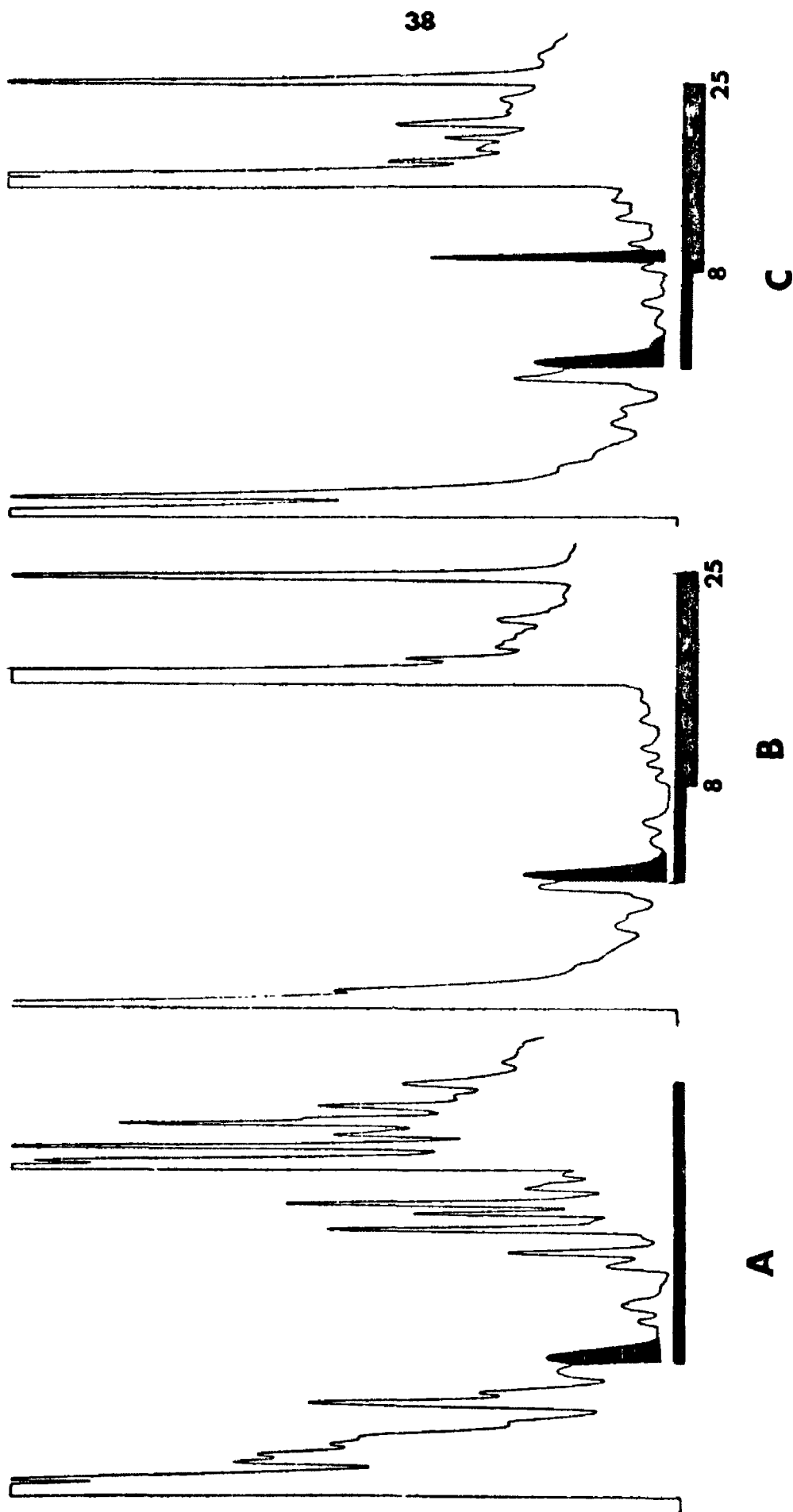
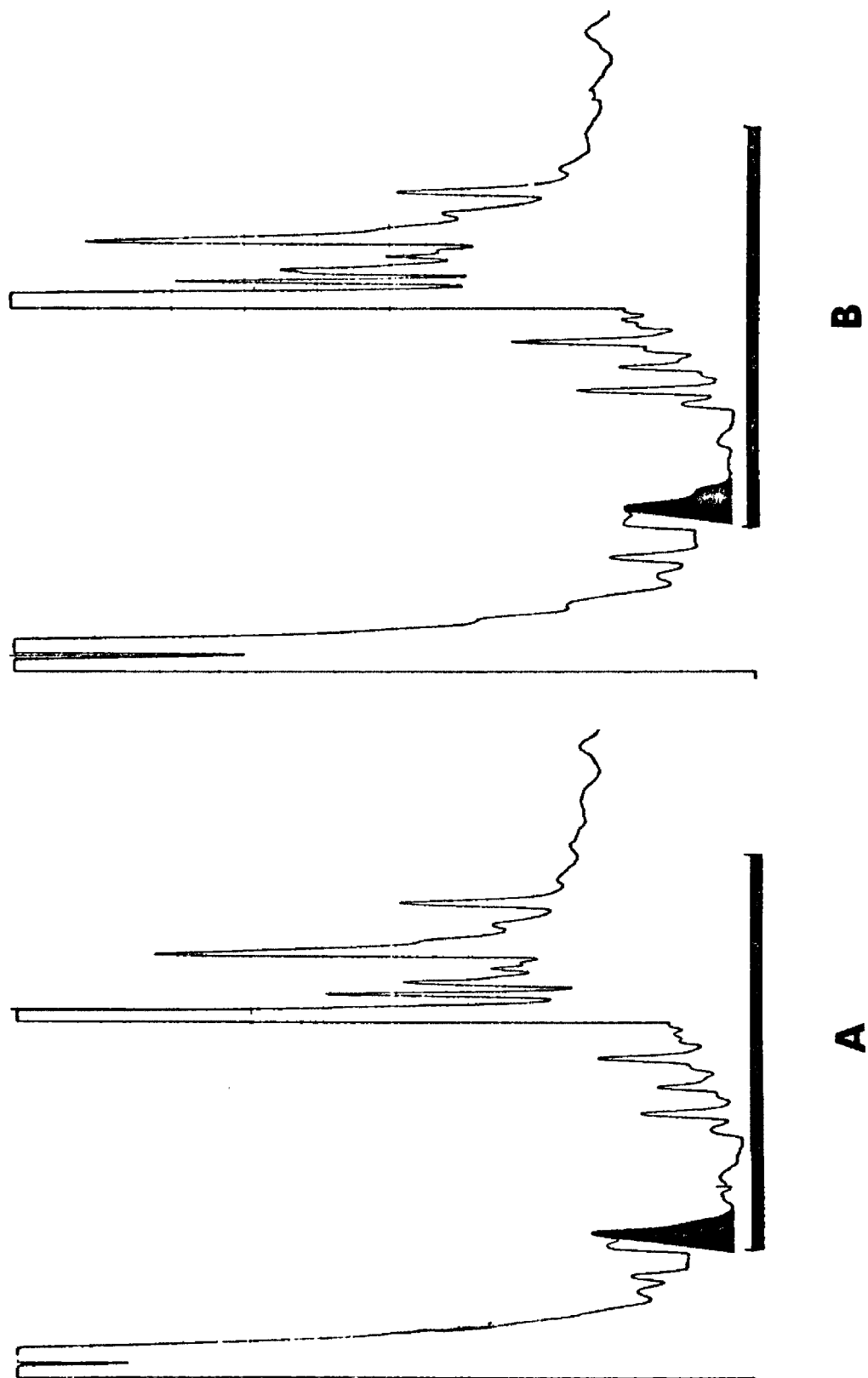


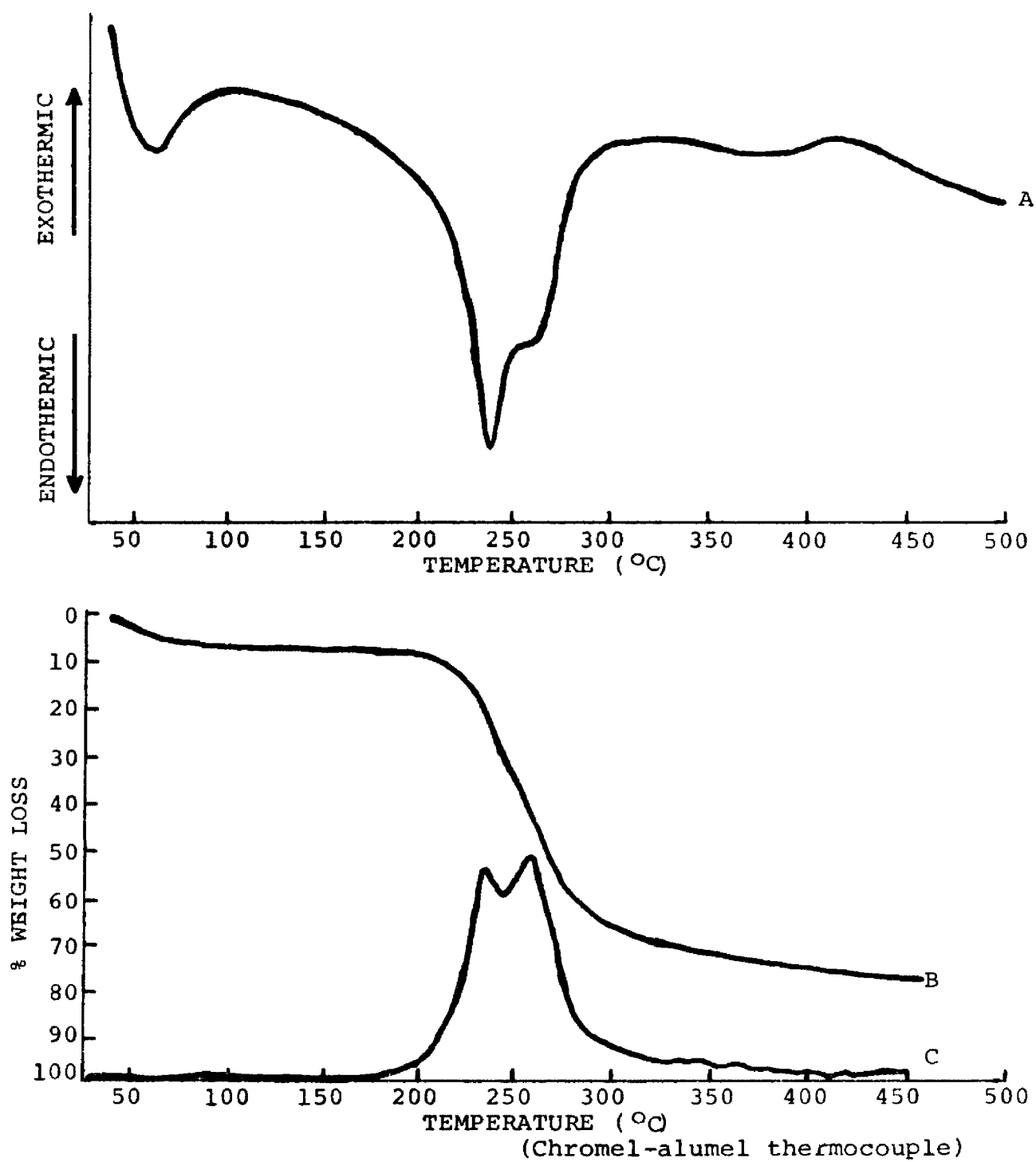
Fig. 10. Inability to differentiate between two strains of E. coli grown for 24 hr in and contained in a simple defined medium. Pyrograms of M-9 + 0.5 % glucose containing a 24 hr culture of E. coli 0113 (A) and a 24 hr culture of E. coli 0111:B4 (B). 200  $\mu$ l of culture was dessicated in a combustion boat and pyrolyzed at 500 C.



analysis and since direct analysis of whole cells removed directly from solid media was still a prime consideration in order to capitalize on the inherent speed and convenience of PGLC, it then became necessary to reevaluate the conditions used for pyrolysis of bacterial whole cells. The methods used for this reevaluation were differential thermal analysis and thermal gravimetric analysis. At the same time, studies were undertaken to ascertain the chemical composition of pyrolysis peaks relative to compounds of known chemical composition found previously to occur in effluent from pyrolysis of carbohydrates.

The mechanism and information obtainable from DTA and TGA have been described in Materials and Methods. Figure 11 (A and B) shows results of DTA and TGA performed on LPS from E. coli 0113. The significance of DTA analysis of LPS is found in the single endothermic deflection which is centered at 250 C. This dip is the result of thermal decomposition of LPS, and the endothermic nature of this deflection indicates that the reaction requires an input of heat. The chemical nature of this reaction will be discussed at a later time, but it should be noted that this major dip at 250 C is the only significant deflection shown by DTA of LPS and no further reactions were detected. Also, it should be noted that the reaction indicated by this deflection was essentially complete by 300 C. TGA data shown in Figure 11 (B) provided additional information about this reaction

Fig. 11. Differential thermal analysis (A), thermal gravimetric analysis (B), and differentiation of thermal gravimetric analysis (C) of LPS from E. coli 0113.



occurring at 250 C. Based on DTA alone, this deflection could have resulted from the decomposition of the sample, molecular rearrangement, formation or destruction of a crystalline structure, or any one of a number of other phenomenon known to occur during pyrolysis (25). However, TGA shows that the sample is undergoing a steady weight loss over the temperature range containing the endothermic dip from DTA. This seems to indicate that the dip is not due solely to chemical rearrangement within the sample, but that it indicated a decomposition accompanied by the release of volatile products into the environment as pyrolysis effluent. The significance of this information for pyrolysis of bacterial materials was that decomposition of the LPS sample was essentially completed by 300 C and that pyrolysis could be carried to completion at a much lower temperature than was used previously. When pyrolysis at 350 C was carried out on bacterial whole cells removed from the surface of solid medium, it was found that a level of resolution was obtained which was not achieved at 500 C. The reasons for this increased resolution at 350 C will be discussed later, but present TGA and DTA data did show that there was little loss of potentially useful pyrolysis effluent due to incomplete sample decomposition at lower temperatures (350 C).

Presumptive information on the chemical composition of peaks resulting from pyrolysis of LPS from E. coli 0113 was obtained by combining known materials with pyrolysis effluent

dissolved in methanol and looking for an increase in the size of a single effluent peak. Figure 12 shows a composite of chromatograms resulting from GLC analysis of 5-methyl furfural (a), GLC analysis of condensed effluent from pyrolysis of LPS of E. coli 0113 dissolved in methanol (B), and GLC analysis of condensed effluent from E. coli 0113 dissolved in methanol containing 5-methyl furfural (C). The shaded peak (Figure 12, C) shows an increase in peak area due to addition of 5-methyl furfural. This peak had a retention time of 2093 seconds which compared with the retention time of 2020 seconds for 5-methyl furfural alone. The difference in retention times represents a discrepancy of approximately 2% of the total development time which is within observed variation in retention times of identical samples due to changes in carrier gas flow and oven temperatures. The conclusion from this data is that the peak in peak region 23 can be tentatively identified as 5-methyl furfural on the basis of retention time and peak enhancement.

Retention times from direct GLC were determined for several other materials and these times are given in Table 1 along with the peak region (cf Figure 3) which would have an approximate correspondence in retention time. Although no effort was made to determine chemically the correlation between these known materials and peaks resulting from pyrolysis of bacterial products, this data indicated what types of materials might be found in the peak regions based



Fig. 12. Peak identification in pyrograms of LPS effluent. The following peaks are shown: 5-methyl furfural (A), effluent from pyrolysis of LPS from E. coli 0113 dissolved in methanol (B), and effluent from pyrolysis of LPS from E. coli 0113 plus 0.01  $\mu$ l 5-methyl furfural placed in a combustion boat and evaporated at 350 C. Shaded peak in (C) indicated that which was increased in area due to addition of 5-methyl furfural.

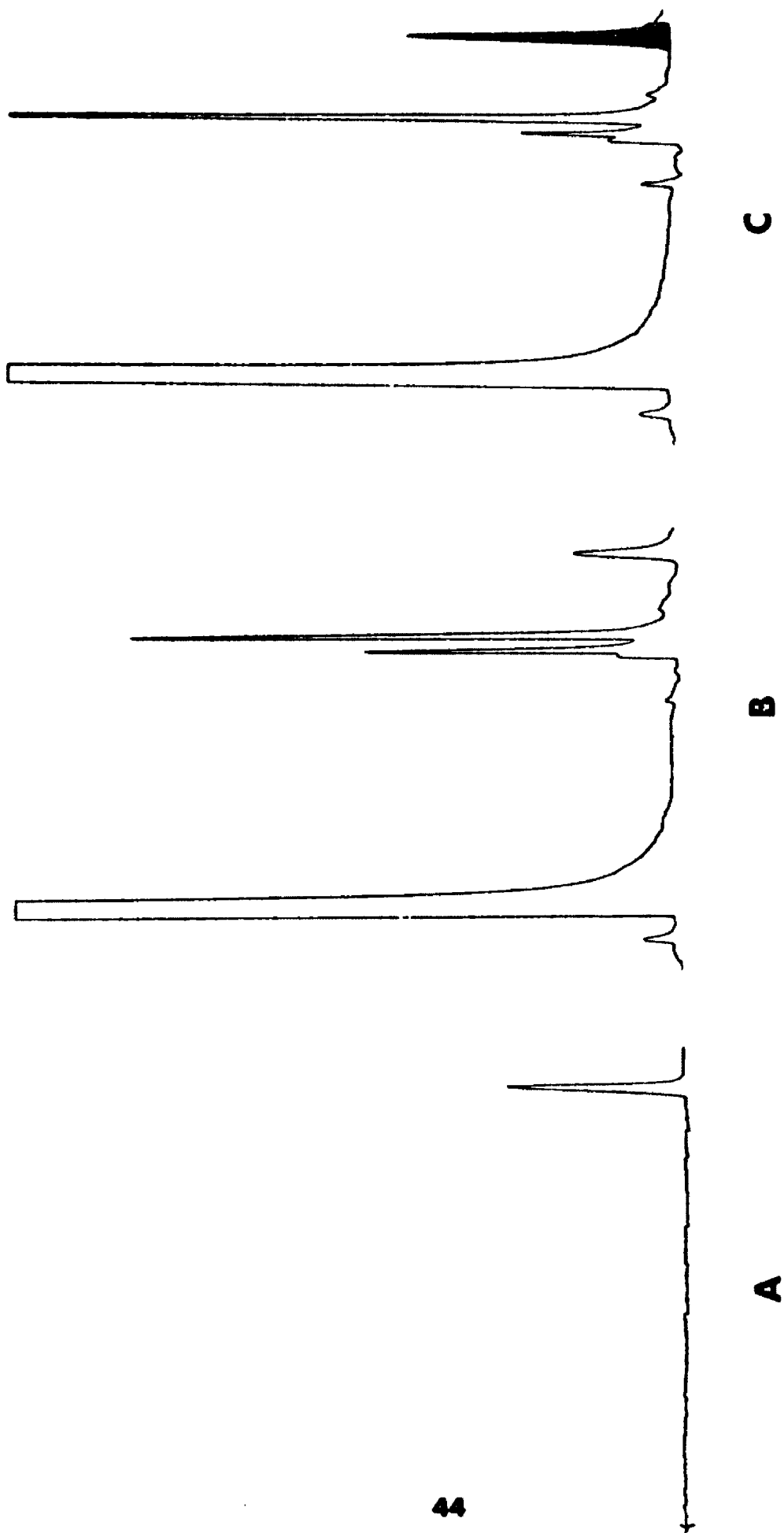


TABLE 1. Retention times for materials which might be found in pyrolyzed sample of LPS or whole cells and their location relative to peak regions from pyrolysis of bacterial whole cells.

Material	Retention Time	Peak Region
Furfural	1740 sec <sup>a</sup>	18
2-Methyl Furan	522	unresolved volatiles <sup>b</sup>
Furan	392	unresolved volatiles
5-Methyl Furfural	2020	23
Acrolein	516	unresolved volatiles

<sup>a</sup>Measured from time of injection of material into pyrolysis oven.

<sup>b</sup>Defined as those areas having retention times between 0 and 900 seconds.

on volatility. Information of this nature could be used eventually to confirm the identity of reference peaks on individual pyrograms prior to statistical analysis.

#### Direct Analysis of Whole Cells from Solid Medium

The real advantages of pyrolytic identification of bacteria are in its rare combination of speed and sensitivity. In order to exploit fully these attributes, it was highly desirable that the time of preparation for samples be kept to a minimum. Ideally, it would be best if cultured whole cells could be removed directly from the surface of complex solid media and pyrolyzed. Although earlier attempts to do this had been unsuccessful, by lowering pyrolysis temperatures from 500 C to 350 C and varying the oven temperature program and carrier gas flow, it was now possible to obtain unique pyrolysis profiles from fresh whole cells recovered from the surface of agar.

Analysis of data from PGLC of fresh whole cells was accomplished in a format which could be adapted to statistical or computer analysis. Bartlet and Smith (2) had shown that the area of both resolved and unresolved chromatographic peaks was related directly to the height of the peak and the width of that peak at a distance 0.882 times the total height measured from the bottom of the peak (the value of the width at this point is equal to the standard deviation). They also determined that the standard deviation increased linearly with an increase in retention time.

Because the area under a peak is, by design, directly proportional to the amount of material giving rise to that peak, and because (according to Bartlett and Smith(2)) that same area can be defined solely on the basis of peak height and retention time, it was felt that peak height and retention time could be used to describe adequately all the information presented in a given pyrogram.

In order to compensate for long term changes in retention time due to column aging and changes in carrier gas flow, the distance between the peak in region 1 and the peak in region 25 was defined as 100%. Distances to the center of all other peaks in a given pyrogram were then measured from peak region 1 and expressed as a percentage of the distance between peak region 1 and peak region 25 on the same pyrogram. These values from different samples of the same strain or species were then averaged and compared to other species and strains (cf Table 2). An examination of this data seemed to indicate that it might be possible to distinguish these particular samples on the basis of the presence or absence of peaks in a given peak region (i.e. the characteristic absence of a peak in peak region 16 on pyrograms of E. coli 0113 and S. typhimurium), but variations between species and strains expressed as percentages of a standard retention time seemed to be due more to fluctuations within the experimental technique rather than to consistent differences in the pyrolysis effluent. This statement is

TABLE 2. Relative retention times from peak regions.

Values for the available samples of each species or strain were averaged and expressed as a percent of the relative distance between peak region 1 and peak region 25. Locations marked (-) indicate that no peak was present in this peak region.

peak region number	percent of standard retention time*				
	<u>E. coli</u> 0113	<u>E. coli</u> 0111:B4	<u>S.</u> <u>typhimurium</u>	<u>S.</u> <u>typhi</u>	<u>S.</u> <u>enteriditis</u>
1	0	0	0	0	0
2	-	7	-	-	-
3	9	10	11	8	11
4	14	15	16	12	17
5	17	19	20	16	17
6	19	21	23	18	23
7	23	26	27	22	28
8	26	28	30	24	31
9	29	33	-	-	-
10	32	34	35	29	37
11	33	36	-	-	-
12	37	40	42	34	43
13	39	42	46	37	45
14	45	48	50	42	52
15	49	52	53	44	55
16	-	54	-	46	57
17	54	56	59	48	59
18	57	61	-	-	-
19	63	65	67	60	65
20	69	71	72	61	73
21	75	76	77	67	78
22	-	83	80	71	83
23	83	-	-	-	85
24	92	92	91	-	91
25	100	100	100	100	100

\*100 % = distance between region 1 and region 25.

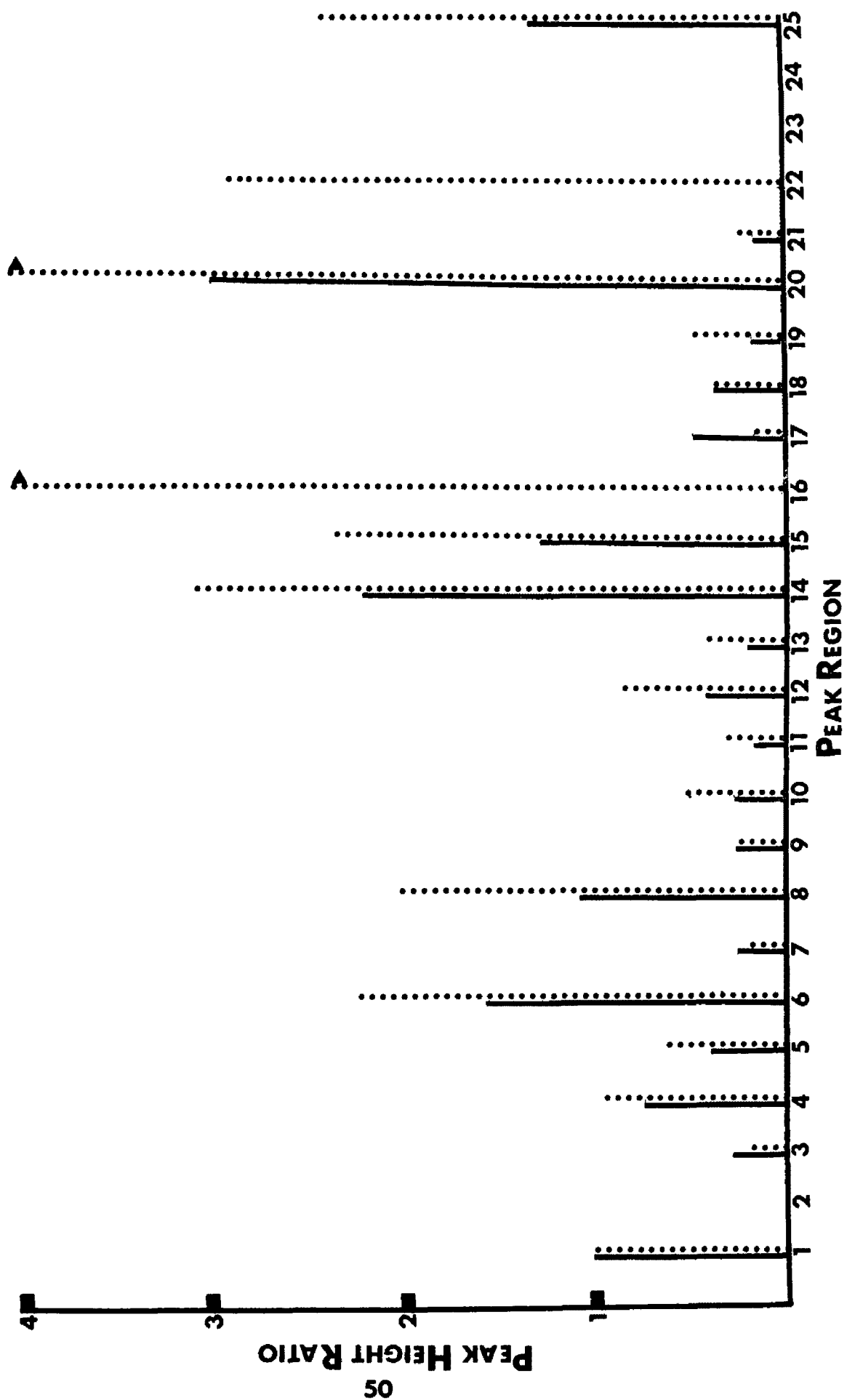
made because of the observed variation between retention times for identical peaks on samples from the same species or strain. This variation was often greater than the observed variation between characteristic peaks used to differentiate pyrograms from different species or strains.

Peak heights were equalized for the differing sample sizes by setting the height of the peak in region 1 equal to 1. Peaks in all other peak regions of a given pyrogram were expressed as a ratio of their height of the peak in region 1 on the same pyrogram. As with retention times, peak height ratios from different samples of the same strain or species were averaged and compared to other species and strains.

Figure 13 is a composite graph showing peak height ratios for each of 25 peak regions. Each peak region contained a peak height ratio averaged from two samples of E. coli 0111:B4 and a relative peak height averaged from three samples of E. coli 0113. Examination of this graph suggested that peak regions 16 and 22 could be used to differentiate pyrograms of E. coli 0111:B4 from pyrograms of E. coli 0113. The distinction was made by noting that these regions in the graph of E. coli 0111:B4 contained peaks having a peak height ratio greater than 2.5 whereas these same regions did not contain peaks in the graph of E. coli 0113. Minor variations in other peak regions (i.e. peak regions 14 and 15) could be used to confirm preliminary distinctions between



Fig. 13. A comparison of pyrolysis profiles, analyzed by peak height ratios, from whole cells of two strains of E. coli. Graph shows relative peak height for E. coli 0111:B4 and E. coli 0113. Values were obtained by averaging peak height ratios for three samples of E. coli 0113 and two samples of E. coli 0111:B4. Lines terminating with a triangle indicate that averaged values exceeded recorder limits.



these two strains but they were not always reliable due to fluctuations between samples of the same strain in these regions (see Figures 15 and 16).

Figure 14 contained the same type of data as presented in Figure 13 except that peak height ratios in each peak region were taken from averaged peak height ratios from two samples each of S. enteriditis, S. typhimurium, and S. typhi. This graph indicated that peak regions 14, 16, and 22 are of primary importance in separating the three species of Salmonella which were pyrolyzed. Comparison of the data on this graph showed that S. typhimurium is the only species showing a peak in peak region 22 with a peak height ratio greater than 1. S. enteriditis could be differentiated from S. typhi by observing the ratio of peak 14 to peak 16. If this ratio was less than 1, the sample graphed was S. enteriditis. In this way, three Salmonella species and two E. coli strains could be separated and identified by pyrolyzing whole cell samples from the surface of nutrient agar, graphing the peak regions in terms of peak height ratios, and comparing the peak height ratios in certain characteristic peak regions.

Figure 15 through 19 were included to show that, in those peak regions which were used to differentiate graphs of the various species and strains, variations between peak height ratios within the same peak region and the same species or strain were always less than variations between

Fig. 14. A comparison of pyrolysis profiles, analyzed by peak height ratios, from whole cells of three species of Salmonella. Graph shows peak height ratios for S. enteriditis, S. typhimurium, and S. typhi. Values were obtained by averaging peak height ratios for two samples of each of the three species. Lines terminating with a triangle indicate that averaged values exceeded recorder limits.

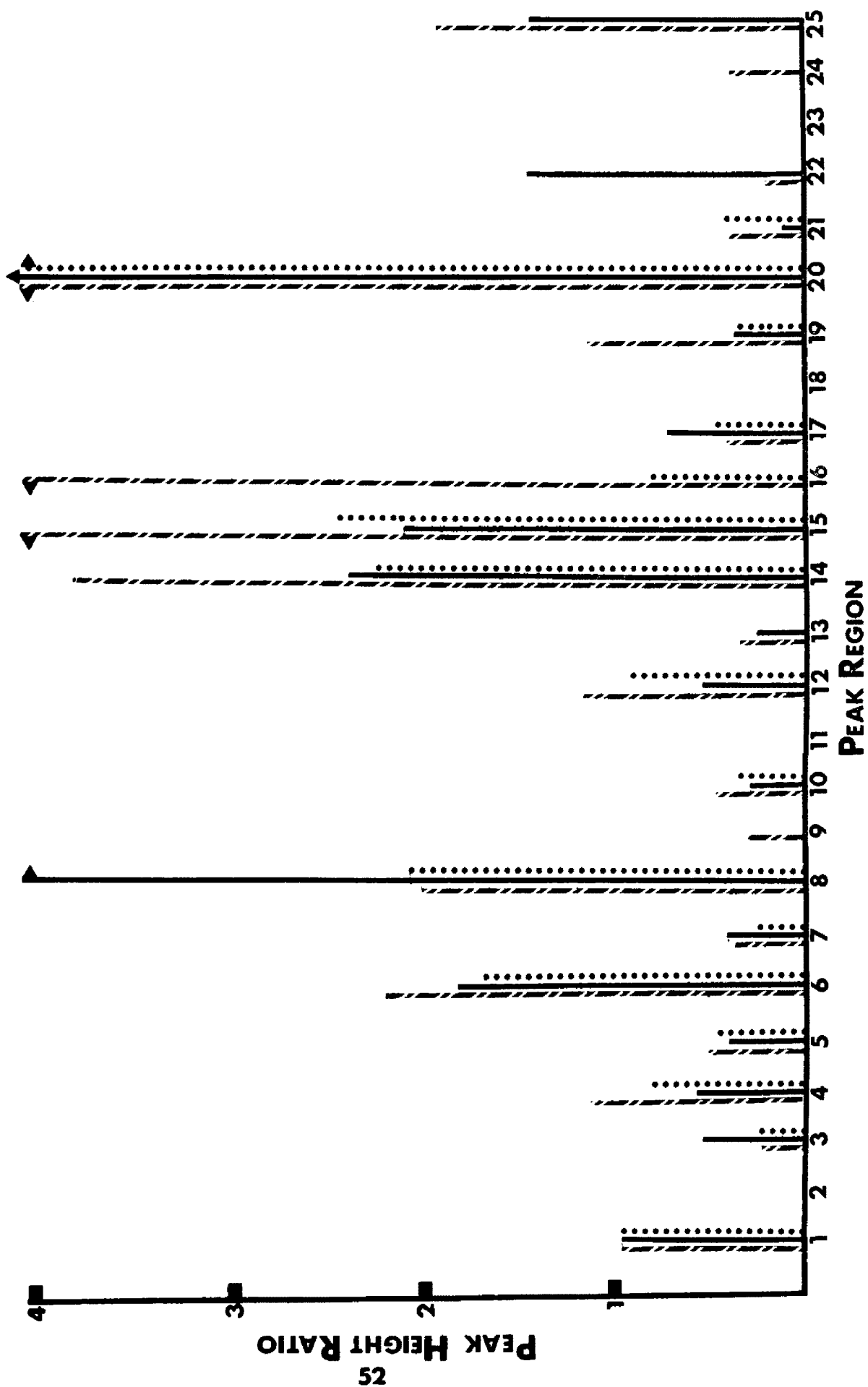
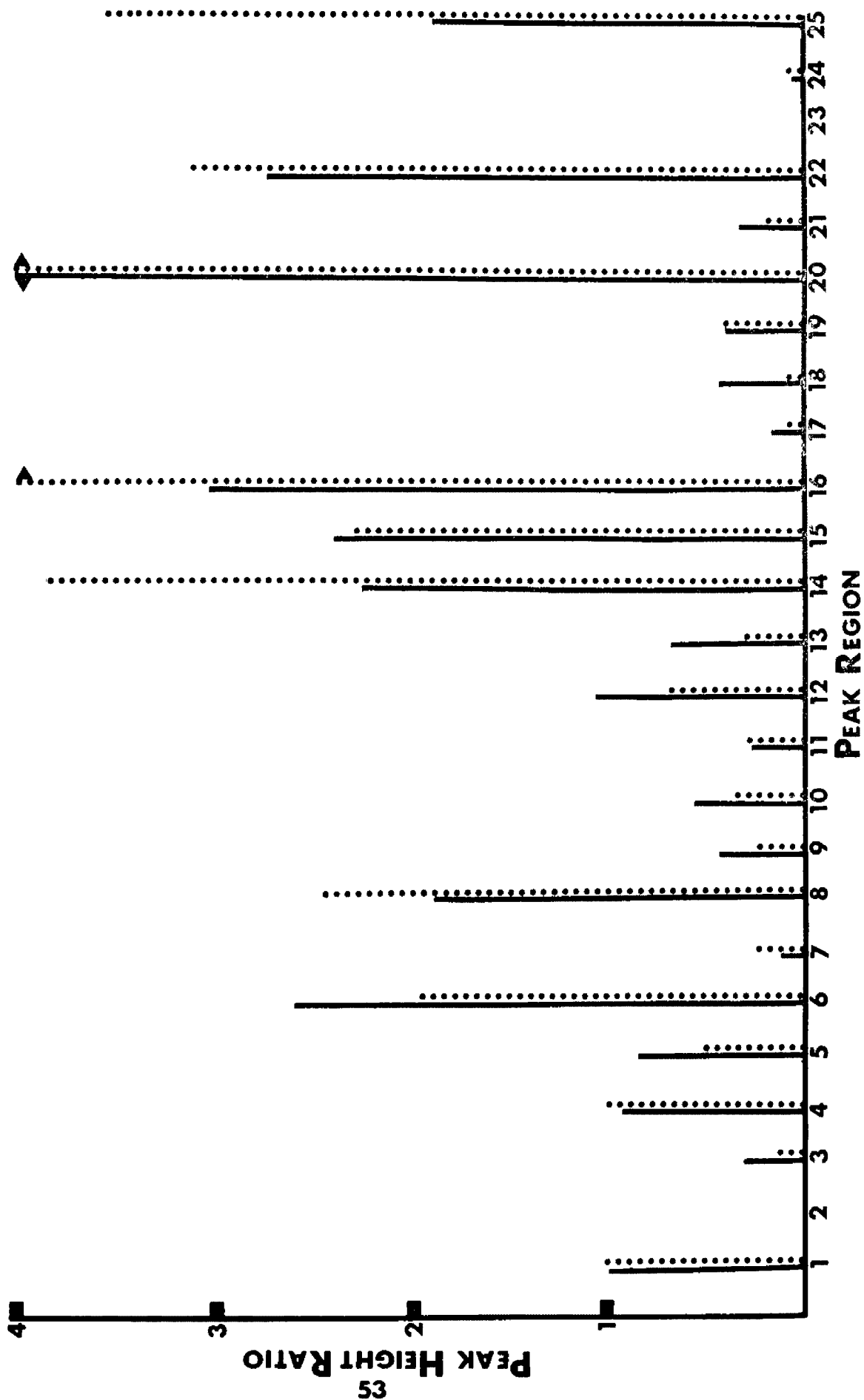
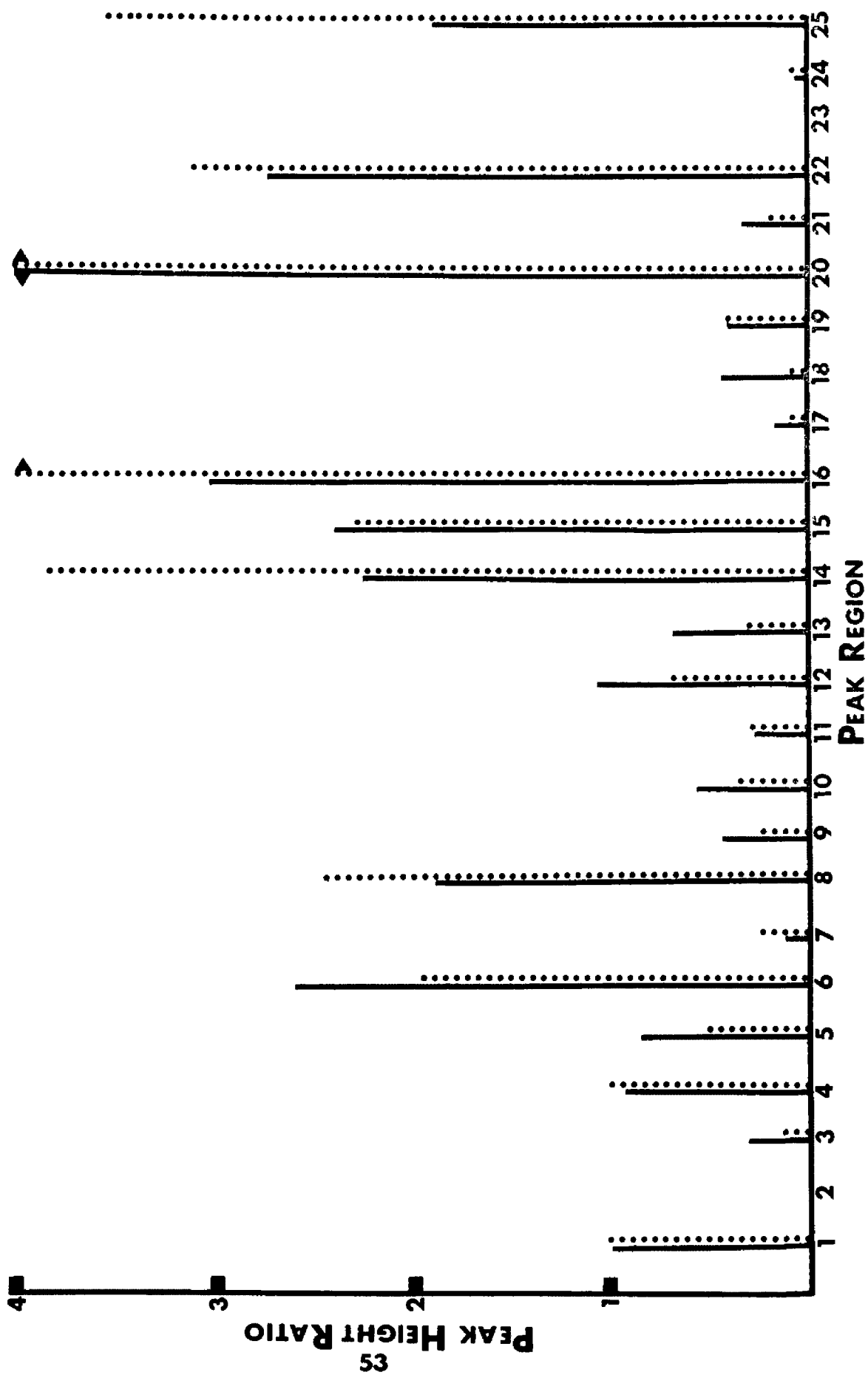


Fig. 15. Reproducibility of pyrograms from individual samples of whole cells from E. coli 0111:B4. Figure shows peak height from each of 25 peak regions divided by peak height from region 1. Lines terminating with a triangle indicate that values exceeded recorder limits.







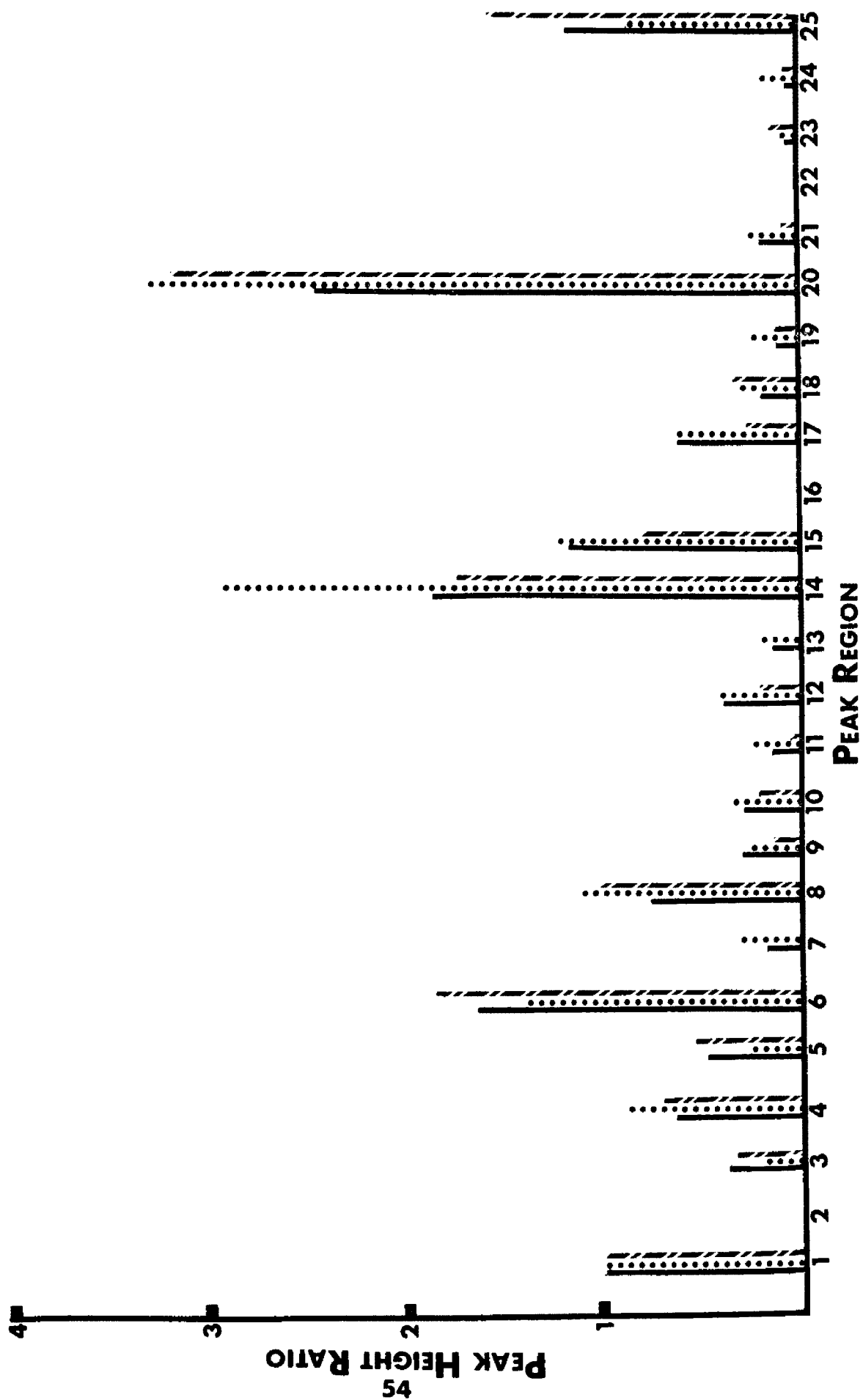


Fig. 16. Reproducibility of pyrograms from individual samples of whole cells from E. coli 0113. Figure shows peak heights from each of 25 peak regions divided by peak height from region 1. Lines terminating with a triangle indicate that values exceeded recorder limits.

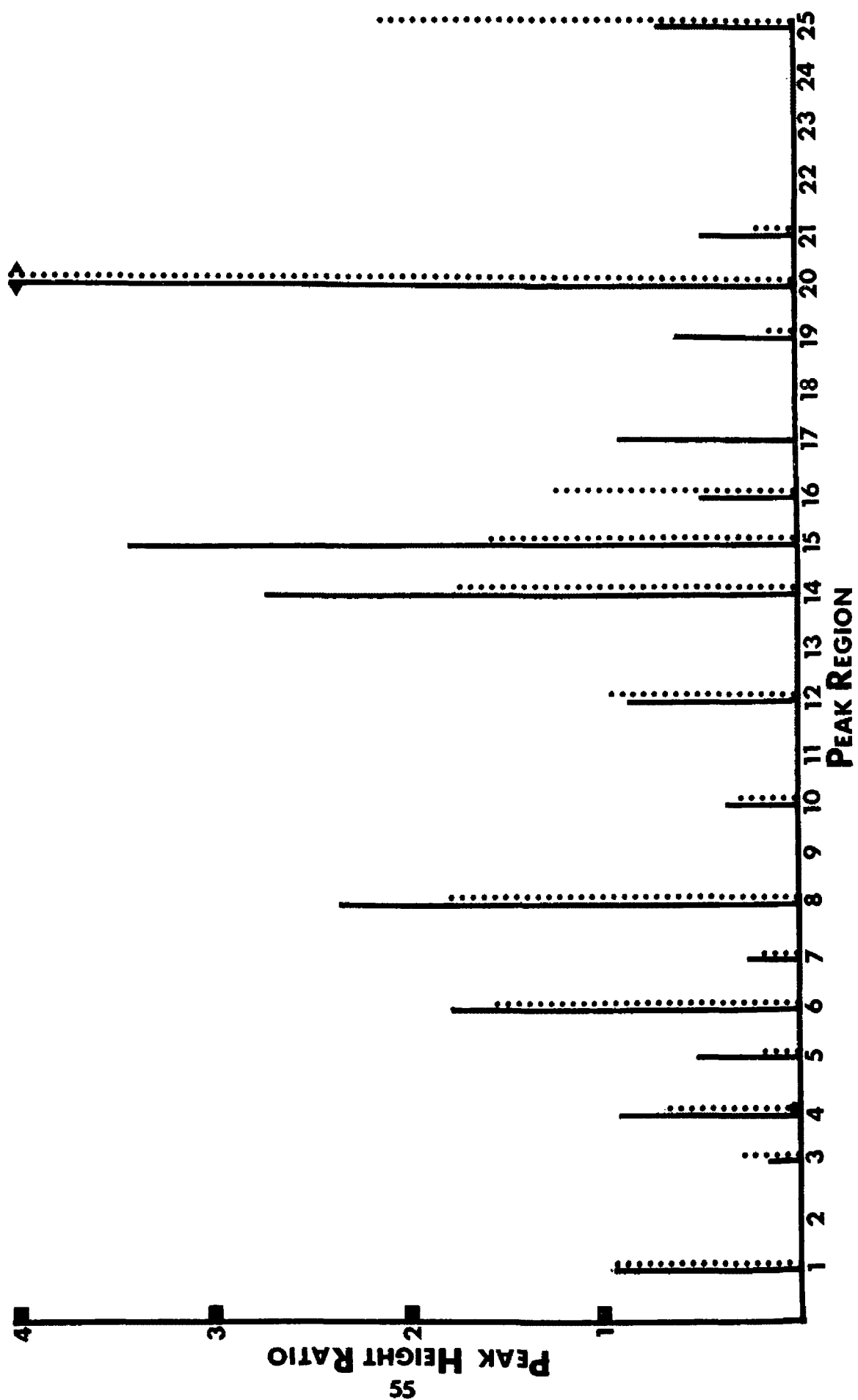


Fig. 18. Reproducibility of pyrograms from individual samples of whole cells from Salmonella typhimurium. Figure shows peak heights from each of 25 peak regions divided by peak height from region 1. Lines terminating with a triangle indicate that values exceeded recorder limits.

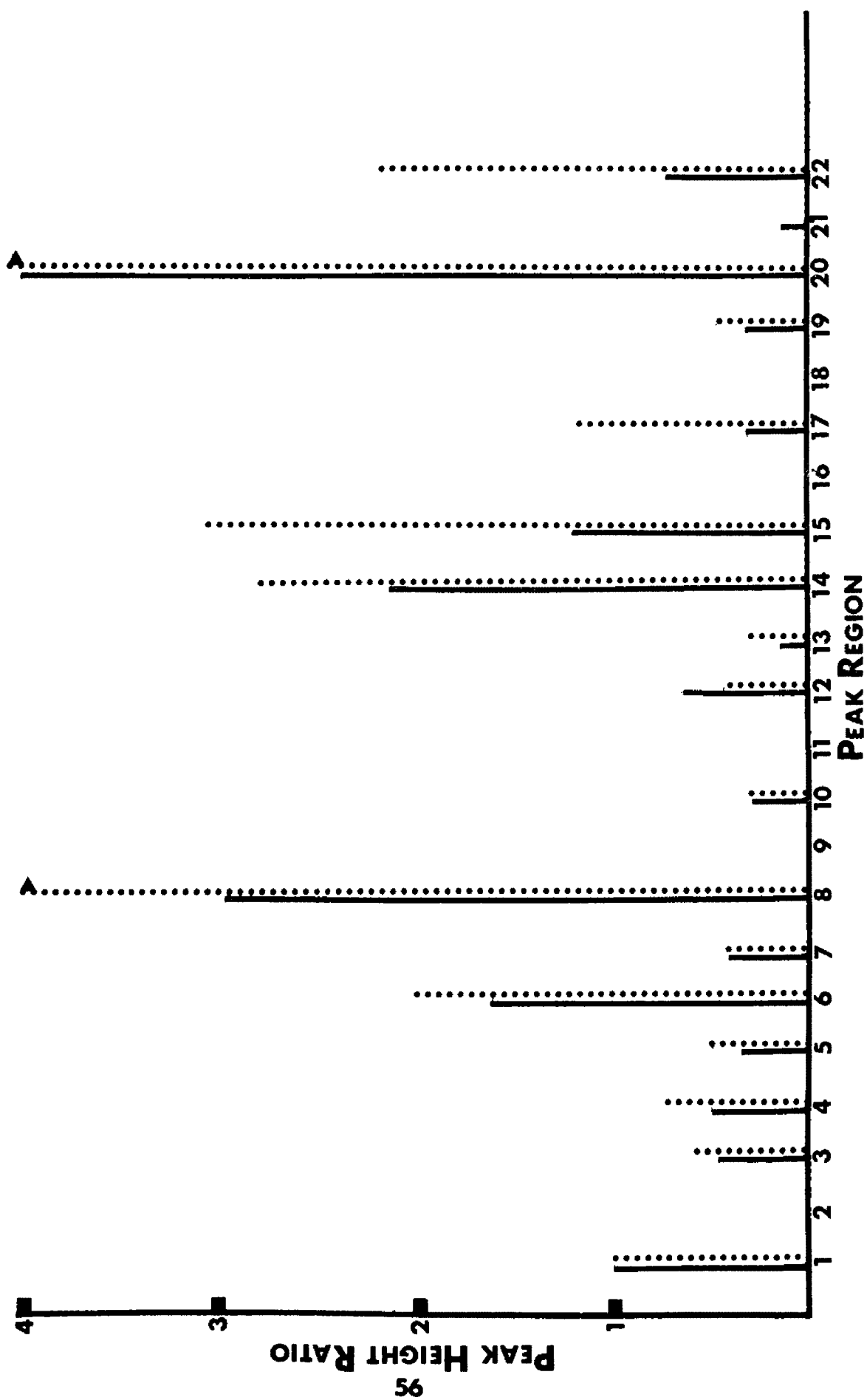
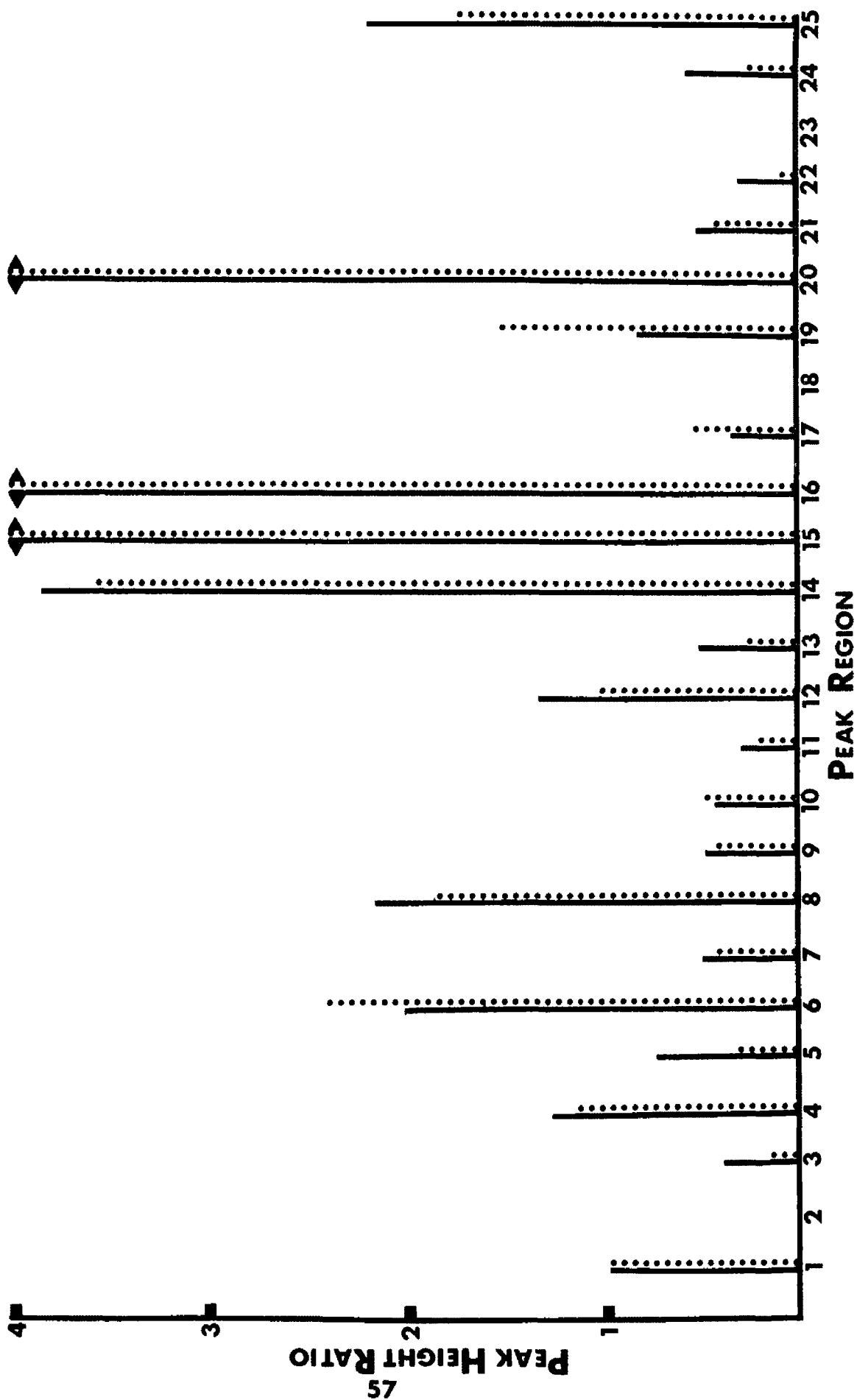


Fig. 19. Reproducibility of pyrograms from individual samples of whole cells from Salmonella enteriditis. Figure shows peak heights from each of 25 peak regions divided by peak height from region 1. Lines terminating with a triangle indicate that values exceeded recorder limits.



peak height ratios within the same peak region on different species or strains. For example, Figure 15 showed that all samples of E. coli 0111:B4 produced peaks in peak region 22 with peak height ratios greater than 2.5. Figure 16 showed that no sample of E. coli 0113 produced any peak in peak region 22. Figure 18 showed that both samples of S. typhimurium produced peak height ratios greater than 0.5 in peak region 22. These same comparisons were made for other significant peak regions to determine whether they would be useful in characterizing the organisms tested.

The presence of peaks which consistently appeared on each pyrogram, and the extence of certain peak regions which could be used to separate organisms on the basis of the peaks within these regions, suggested that a system of computer identification might be set up based on the use of reference peaks for indexing and selected peak regions for differential identification. Table 3 was formulated to show how this might be done. Taking the values for averaged peak height ratios from this table, the computer could be programed to process only those peak regions which were known to be significant. In the case of analysis involving separation of E. coli 0111:B4 and E. coli 0113, the peak regions processed would be 16 and 22. The computer could then determine whether the peak height ratio in a certain peak region exceeded a specified value or whether the ratio of peak height ratios in two peak regions exceeded a speci-



TABLE 3. A numerical representation of average relative peak heights from two strains of E. coli and three species of Salmonella, expressed in numerical format. Values marked with parenthesis were used for primary characterization and differentiation of these organisms.

peak region number	Averaged peak height ratios*				
	<u>E. coli</u> 0113	<u>E. coli</u> 0111:B4	<u>S.</u> <u>typhimurium</u>	<u>S.</u> <u>typhi</u>	<u>S.</u> <u>enteriditis</u>
1	1.000	1.000	1.000	1.000	1.000
2	0	.040	0	0	0
3	0.299	0.196	0.523	0.208	0.207
4	0.771	0.964	0.618	0.743	1.141
5	0.423	0.849	0.413	0.448	0.482
6	1.593	2.286	1.817	1.700	1.970
7	0.236	0.175	0.394	0.211	0.372
8	0.989	2.187	3.466	2.086	1.750
9	0.227	0.313	0	0	0
10	0.250	0.455	0.274	0.334	0.452
11	0.124	0.269	0	0	0
12	0.440	0.870	0.540	0.825	1.179
13	0.181	0.487	0.207	0.080	0.077
14	21214	3.097	2.468	(2.242)	(3.585)
15	1.076	2.351	2.227	2.476	>4.000
16	(0)	3.050	0	(0.937)	(>4.000)
17	0.380	0.144	0.792	0.490	0.413
18	0.261	0.651	0	0	0
19	0.149	0.391	0.396	0.383	1.144
20	3.024	>4.000	4.365	>4.000	>4.000
21	0.141	0.242	0.093	0.382	0.373
22	(0)	(2.954)	(1.445)	(0.080)	(0.206)
23	0.070	0	0	0	0.063
24	0.096	0.150	0.091	0	0.390
25	1.317	2.724	2.230	1.000	2.002

\*Peak height from each region divided by peak height from region 1.

fied value. A specific example of the latter would be separation of S. enteriditis and S. typhi based on the ratio of the value in peak region 16 (Table 3). In the case of S. enteriditis, this ratio is less than 1; in the case of S. typhi, it is ca. 2.4. Following this same pattern, mathematical procedures for distinguishing large numbers of organisms could be programed into a computer and used to process both clinical and taxonomic samples.

## CHAPTER IV

### DISCUSSION

#### Observations Relating to Resolution of PGLC

Although in the past, considerable effort has been devoted to the development of techniques suitable for pyrolytic analysis of bacterial and fungal materials (16, 17, 31), most recent studies in this area have been directed primarily towards the use of existing pyrolysis techniques for the examination of additional strains and species of microorganisms (7, 13, 30). Because of difficulties which have been reported during attempts to separate bacteria at the strain level by PGLC (16, 17), it was felt that the inherent resolution of PGLC was either insufficient or had not been refined fully. The purpose of the present study therefore, was to determine how closely bacterial materials could be related chemically and antigenically and still be resolved by PGLC. In order to do this, the standard techniques for pyrolysis of these materials were modified and experimental conditions were refined so that only the basic concepts of pyrolysis were retained.

Several factors were found to effect substantially the resolution obtainable by PGLC of bacterial materials. These factors included the temperature at which pyrolysis

was carried out, the amount of sample pyrolyzed relative to the sensitivity of the detectors, the carrier gas flow rate, and the temperature programming of the column oven. At various times during the project, these items were adjusted empirically with the goal of obtaining increased resolution between pyrograms of closely related materials. In some cases equipment was modified in order to improve performance. An example of the latter was valving of the carrier gas supply to the sample column which shunted the flow of carrier gas around the pyrolysis oven during the development of the pyrogram.

The one variable which seemed to have the greatest effect on the amount of resolution afforded by PGLC was the temperature at which pyrolysis was carried out. Because of this observation, special consideration was given to studies on the effect of temperature on pyrolytic reactions. Previous work on the effects of pyrolysis temperature on effluents from carbohydrates had been carried out by Shafizadeh et al. (24) in studies on cellulose, levoglucosan and phenyl B-D-glucopyranoside. It was observed that temperature markedly affected the pyrolytic process both qualitatively and quantitatively. Increased formation of fixed gases ( $\text{CO}$ ,  $\text{CO}_2$ ,  $\text{C}_2\text{H}_4$ , and  $\text{C}_2\text{H}_6\text{O}$ ) was observed when the pyrolysis temperature was increased from 600 C to 700 C. Also, the amount of organic volatile products increased when pyrolysis temperature was raised from 500 C to 600 C. A change from 600 C

to 700 C resulted in a four-fold increase in volatile products. The amount of fixed gases increased sixteen-fold with the same 600 C to 700 C increase in temperature. The conclusion was reached that raising temperatures into the 600 C to 700 C range increased secondary pyrolytic reactions, and these reactions resulted in multiple fragmentation products having low molecular weights characteristic of fixed gases and volatile organic products (2 to 5 carbon materials). In another study involving slow heating (20 C per day) of cellulose (25), it was found that the crystalline structure of the cellulose molecule remained intact up to a temperature of 250 C. At temperatures exceeding 250 C, the results of infrared studies showed that the crystalline structure was destroyed by cleavage of the oxygen ring and formation of carboxyl groups.

It is a relatively long step to relate the data obtained from cellulosic materials to data obtained from bacterial materials, however, DTA analysis of LPS from E. coli 0113 did provide some interesting correlations. In particular, it was noted that DTA analysis of LPS showed a major deflection centered at 250 C and this dip indicated endothermic decomposition. Whether this deflection in the DTA pattern from LPS was due to the same reaction as found in cellulose at 250 C is unknown, but it seems possible that the same type of transglycolation reactions take place which are responsible for depolymerization of cellulose (26)

in the polysaccharide portion of LPS. As further evidence of the relationship between pyrolysis of cellulose and bacterial LPS, TGA data from LPS correlated well with the TGA data from cellulose in that both materials showed a rapid weight loss at approximately 250 C (25).

The significance of a possible correlation between pyrolysis of plant cell wall polysaccharides and pyrolysis of bacterial materials was that pyrolysis of cellulosic materials at higher temperatures (300 C and above) probably led to further degradation of primary pyrolysis products. These reactions which took place at higher temperatures gave rise to the predominance of fixed gases and volatile organic materials. If similar reactions occurred during high temperature pyrolysis of bacterial materials, it would be possible that these same fixed gases and volatile organic materials would have been produced due to decomposition of intermediate pyrolytic products. Since it was shown that high temperature pyrolysis of cellulose, levoglucosan and phenyl  $\beta$ -D-glucopyranoside led to very similar pyrolysis elution patterns, it was possible that unique intermediate products of pyrolytic decomposition were being decomposed further by reactions which formed products common to pyrolysis of many organic materials. If high temperature decomposition were predominating during pyrolysis of bacterial materials, any resolution due to unique intermediate pyrolysis products would be lost or

reduced at pyrolytic temperatures high enough to cause further decomposition.

In support of this theory, it was found that when the temperature employed for pyrolyzing LPS was increased from 350 C to 500 C, a decrease in the total number of peaks occurring between GLC retention times of 0 and 900 seconds was noted. This corresponded to a conversion of the intermediate products of pyrolysis into volatile gases (i.e. CO<sub>2</sub>, CO, CH<sub>3</sub>). When pyrolysis was carried out at 350 C, resolution between LPS from E. coli 0111:B4 and E. coli 0113 was improved over that obtained at 500 C. This resolution was noted by comparing differences in both location and size of characteristic peaks in peak regions 1 through 25.

#### Separation of Bacterial Materials

Studies focusing on the ability of PGLC to distinguish among antigenically related bacterial materials were initiated with extracts of some chemically defined polymers and the bacterial whole cells from which these extracts were obtained. This system consisted of NPP, LPS, and whole cells from E. coli strains 0113 and 0111:B4. Because LPS from E. coli 0113 and E. coli 0111:B4 did not cross-react antigenically (20, 21), this pair of bacteria and their extracts met the criteria for an antigenically defined model with which to evaluate the resolution of PGLC under controlled conditions. Also, the chemotype of E.



coli 0111:B4 had been worked out (11) and could be used for studies pertaining to the mechanisms of pyrolysis.

In the initial studies, it was possible to identify pyrograms of LPS extracted from the two closely related strains of E. coli. Therefore, it was concluded that PGLC had resolution sufficient to distinguish LPS extracts at the strain level. It might be assumed that differences in the polysaccharide portion of the LPS [which have been shown to account for the antigenicity of LPS (11)] might also account for the resolution shown by PGLC, but this was not confirmed. At this point, it was felt that the resolution afforded by PGLC was adequate for extracted LPS; however, the usefulness of PGLC for rapid identification of bacteria would be dependent on the development of simple extraction procedures. As an alternative, the procedure could be modified to allow direct analysis of bacterial whole cells.

Simple extraction procedures were examined first with extraction accomplished by boiling whole cells in water. This was successful and resulted in a material which gave pyrolysis elution patterns similar to those from purified LPS. Using this technique, it was possible to place the supernatant fluid from a solution of boiled cells into a combustion boat, evaporate this material, and then reintroduce an additional sample for evaporation. In this way, sufficient material could be obtained in order to give

a useable pyrolysis pattern. PGLC of these preparations resulted in resolution comparable to that afforded by LPS extracted by classical procedures. Although use of this extraction procedure was not as convenient and rapid as direct pyrolysis of fresh whole cells, this method might prove useful in cases where direct pyrolysis of whole cells did not afford adequate resolution.

Work with various growth media and cell preparation procedures pointed out some difficulties inherent in the pyrolytic identification of bacteria in cultures. Pyrolysis of sterile growth media such as TSB and M-9 + 0.5 % glucose yielded pyrograms which were identical to pyrograms of the same media containing 24 hr cultures of bacterial whole cells. As discussed earlier, the problem posed by secondary pyrolytic reactions at higher pyrolysis temperatures (500 C to 700 C), was felt to be a major factor in the lack of resolution obtained from bacteria grown in complex liquid media. It was proposed that the medium in which the cells were grown gave pyrolysis patterns which masked the unique patterns from the bacterial cells. This problem could be circumvented by washing the cells prior to lyophilization, but this required an additional step. When M-9 medium with glucose was used, it was possible to achieve resolution between E. coli 0113 whole cells and E. coli 0111:B4 whole cells. This resolution was possible only if the cells were cultured in the synthetic medium

for 48 hr. Conceivably, this increase in resolution occurred because the cells utilized most of the glucose during the 48 hr period and also secreted their own characteristic metabolic by-products into the medium. Pyrolysis of M-9 + glucose gave a pyrolysis elution pattern characteristic of glucose pyrolyzed alone. This pattern was not present when a 48 hr culture of lyophilized M-9 plus bacterial whole cells was pyrolyzed and was replaced by a pattern unlike either M-9 plus glucose or washed whole cells.

One of the most promising series of results provided by this investigation was obtained by pyrolyzing whole cells removed from the surface of solid media. The high level of resolution achieved between whole cells of E. coli 0111:B4 and E. coli 0113 was particularly encouraging because of the implications that other closely related organisms could be distinguished by PGLC with a minimum of preparation prior to pyrolysis. Adequate resolution between whole cells was achieved only after varying the processes of pyrolysis. Further increases in resolution might be obtained by additional studies on variations in the pyrolytic process. Of particular interest would be the ability to decrease GLC development time by eliminating the need to record those portions of the pyrogram which do not contribute to its differentiation from other pyrograms. This could be done by either manipulating the temperature program of the column oven or by choosing columns which would selectively separate

only the effluent responsible for resolution. This selective separation would be particularly desirable since observations made during the present investigation indicated that only the middle 1/3 of a completed pyrogram was useful in differentiating among pyrograms from other organisms. Unfortunately, the other portions of the pyrogram must somehow be eluted in order to clear the columns for subsequent assays.

#### Quantitative Treatment of Data

In order to deal quantitatively with the data provided by pyrolysis of whole cells from solid media, an effort was made to identify certain effluent peaks which might be readily identified on individual pyrograms and used as reference peaks. Simmonds (30) had established the feasibility of qualitative analysis of pyrolysis products by spectroscopy of pyrolysis elution patterns from lyophilized bacterial whole cells. Although these procedures may have a place in subsequent bacterial pyrolysis studies, in this current study it appeared that identification of a relatively small number of peaks by simpler techniques would be more valuable in establishing reference peaks.

In general, results of studies on peak identification showed that certain peaks could be tentatively identified by peak enhancement with known substances and by their relative retention times. The peaks that were identified were not used as reference peaks because they could not be

located on all the pyrograms examined. In lieu of a reference peak which could be chemically identified, a reference peak was chosen which could be visually identified (based on retention time and size) on each pyrogram examined. This reference peak was used subsequently as a standard by which variations in retention times and peak areas due to variations in experimental conditions could be determined and compensated for.

The final portion of this study was to examine how PGLC data might be adapted to a system of numerical taxonomy and computerized identification of microorganisms. The foundation of such a system would consist of PGLC data obtained so as to achieve maximum reproducibility. Techniques for obtaining this degree of reproducibility would include rigid control of pyrolysis temperature, sample size and condition of preparation, oven temperature programming, carrier gas flow rates, column condition, and detector sensitivity. In addition, the output from the detectors would be expressed in a numerical format by electronic integration of peak areas.

Once peak areas from PGLC had been determined quantitatively, they would be assigned to peak regions based on retention times. The retention times used for these assignments would be normalized by the computer relative to the retention times of specific reference peaks and would thus be adjusted for slight variations in experimental conditions.

Evaluation of the peak regions for identification of the organism could be accomplished either sequentially or simultaneously. If done sequentially, the computer would evaluate significant peak regions according to a prescribed schedule and results of each evaluation would determine the next step in the sequence. For example, if the peak area in peak region 22 was less than a predetermined value, a determination of the ratio of the peak area in region 24 to the peak area in peak region 17 might be the next step in the sequence. If the area was greater than this predetermined value, the computer would be instructed to enter the area in peak region 16 as the next step. Each step in the sequence would also eliminate certain organisms from consideration in the identification of the unknown. Disadvantages of the sequential method are that it is slow when compared to simultaneous procedures (however, this is not really a factor in computer evaluation) and a single error at one step in the sequence would give an incorrect identification regardless of the validity of previous or subsequent results.

The simultaneous method would probably be most applicable to computer analysis of PGLC data. In this method, the data bank would consist of all organisms in a specified group (i.e. all Gram-negative enteric bacteria) and the average peak area in each peak region for each organism (as determined from known samples). The computer would then

take the area in each peak region from the unknown and compare it to peak areas for that peak region as contained in the data bank. Methods for statistical analysis of the results of this comparison can be found in several sources (3, 8, 32) and will not be discussed here.

The types of analysis suggested here would be of limited use in a clinical laboratory where equipment, time and a limited requirement for differentiating large numbers of closely related organisms would render its use impractical. However, for taxonomic studies or large diagnostic laboratories, the need for rapidly classifying closely related organisms would make computer linked PGLC a valuable tool. Average time for a single determination from whole cells would be approximately one hour. This might be decreased by selecting for those portions of the pyrolysis elution pattern containing significant peaks. A normal battery of selective and differential media tests would take at least 24 hrs to give results. Serological examinations would require about the same time period. The primary disadvantage of PGLC would be the inability to run many samples simultaneously. Since PGLC would probably be used for the few determinations that could not be made by standard diagnostic procedures, this would not be a major disadvantage and refinements in PGLC to adapt it to this role would be justified.

## CHAPTER V

### SUMMARY

Several materials of bacterial origin were analyzed by pyrolysis and gas-liquid chromatography. These materials included native protoplasmic polysaccharide, lipopolysaccharide, and bacterial whole cells. By using these materials it was determined that PGLC could differentiate purified extracts as simple as LPS (but not NPP) and as complex as whole cells removed from the surface of nutrient agar. This research demonstrated that differentiation was possible when the above materials were derived from two strains of E. coli having a close antigenic relationship. It was also possible to resolve whole cell pyrograms from three species of Salmonella.

It was found that temperature had the most significant effect on resolution and the lowest temperature giving complete pyrolysis was found to yield optimum resolution. Differential thermal analysis and thermal gravimetric analysis were used to find this optimal temperature and to correlate pyrolysis of LPS with pyrolysis studies done on cellulosic materials. Data from this current research and additional information from studies of the pyrolysis of cellulosic materials led to the conclusion that secondary



pyrolysis of primary pyrolytic residues at high pyrolysis temperatures was the reason for decreased resolution noted from pyrograms of bacterial materials pyrolyzed at these temperatures.

A method of data analysis was formulated based on the use of reference peaks appearing on all pyrograms examined. All other retention times and peak heights were compared to those of the reference peaks and data was thus standardized regardless of variations due to column aging, carrier gas flow, and oven temperature. The results from pyrolysis of whole cells was presented in a numerical format and a scheme which could be adapted to computer analysis was proposed for use in large clinical or taxonomic laboratories.

## LITERATURE CITED

1. Anacker, R. L., W. D. Bickel, W. T. Haskins, K. C. Milner, E. Ribí, and J. A. Rudbach. 1966. Frequency of occurrence of native hapten among enterobacterial species. *J. Bacteriol.* 91:1427-1433.
2. Bartlett, J. C., and D. M. Smith. 1960. The determination of the areas of resolved and partially resolved chromatography peaks. *Canadian J. Chem.* 38:2057-2065.
3. Beers, R. J., and W. R. Lockhart. 1962. Experimental methods in computer taxonomy. *J. Gen. Microbiol.* 28:633-639.
4. Bourne, E. J., and H. Weigel. 1965. Extraction with chloral hydrate, p. 78-80. In R. L. Whistler, and M. L. Wolfrom (ed.), *Methods in carbohydrate chemistry*. vol. V. Academic Press, Inc., New York.
5. Braun, V. 1973. Molecular organization of the rigid layer and the cell wall of *Escherichia coli*, p. 1-8. In E. H. Kass and S. M. Wolff (ed.), *Bacterial lipopolysaccharides*. The University of Chicago Press, Chicago.
6. Buchanan, R. E., and N. E. Gibbons (ed.). 1974. *Bergey's manual of determinative bacteriology*. 8th edition. p. 298-300. Williams and Wilkins Company, Baltimore.
7. Cone, R. D., and R. V. Lechowich. 1970. Differentiation of *Clostridium botulinum* types A, B and E by pyrolysis-gas-liquid chromatography. *Appl. Microbiol.* 19:138-145.
8. Friedman, R. B., D. Bruce, J. MacLowry, and V. Brenner. 1973. Computer-assisted identification of bacteria. *Am. J. Clin. Path.* 60:395-403.
9. Hurd, E. D., A. R. Goldsby, and E. N. Osborne. 1932. Furan reactions. II. Furan from furfural. *J. of the Amer. Chem. Soc.* 56:2532-2540.

10. Lüderitz, O., A. H. Staub, and O. Westphal. 1966. Immunochemistry of O and R antigens of Salmonella and related Enterobacteriaceae. Bacteriol. Rev. 30:192-225.
11. Lüderitz, O., O. Westphal, A. M. Staub, and H. Nikaido. 1971. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides, p. 145-233. In G. Weinbau, S. Kadis, and S. J. Ajl (ed.), Microbial Toxins, vol. 4. Academic Press, Inc., New York.
12. Kauffmann, F. 1950. The diagnosis of Salmonella types, p. 3-12. Charles C Thomas, Springfield.
13. Kulik, W. N., and P. G. Vincent. 1973. Pyrolysis gas-liquid chromatography of fungi: Observations on variability among nine Penicillium species of the section Asymmetrica, subsection Fasciculata. Mycopathol. Mycol. appl. 51:1-18.
14. Milner, K. D., J. A. Rudbach, and E. Ribi. 1971. General characteristics, p. 1-65. In G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial Toxins, vol. 4. Academic Press, Inc., New York.
15. Morgan, W. T. J. 1965. Extraction with diethylene glycol, p. 80-83. In Methods in carbohydrate chemistry. vol. V. Academic Press, Inc., New York.
16. Reiner, E. 1965. Identification of bacterial strains by pyrolysis gas-liquid chromatography. Nature, (London). 206:1272-1274.
17. Reiner, E., and W. H. Ewing. 1968. Chemotaxonomic studies of some Gram-negative bacteria by means of pyrolysis gas-liquid chromatography. Nature (London). 217:191-194.
18. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. 1955. In Studies of biosynthesis of Escherichia coli. p. 5. Carnegie Inst. Wash. Publ. 607.
19. Rudbach, J. A. 1974. Personal Communication.
20. Rudbach, J. A. 1970. Molecular immunogenicity of bacterial lipopolysaccharide antigens: Establishing a quantitative system. J. Immunol. 106:993-1001.

21. Rudbach, J. A., K. C. Milner, and E. Ribí. 1967. Hybrid formation between bacterial endotoxins. *J. Expt. Med.* 126:63-79.
22. Rudbach, J. A., K. C. Milner, R. L. Anacker, E. Ribí and W. T. Haskins. 1967. Physical structure of a native protoplasmic polysaccharide from *Escherichia coli*. *J. Immunol.* 98:1-7.
23. Rudbach, J. A., K. D. Milner, and E. Ribí. 1969. Occult endotoxin in bacterial protoplasm. *J. Bacteriol.* 99:51-56.
24. Shafizadeh, F. 1975. Personal Communication.
25. Shafizadeh, F. 1968. Pyrolysis and combustion of cellulosic materials, p. 419-476. In *Advances in carbohydrate chemistry*. vol. XXIII. Academic Press, Inc., New York.
26. Shafizadeh, F., and Y. L. Fu. 1972. Pyrolysis of cellulose. *Carbohydrate Research*. 29:113-122.
27. Shafizadeh, F., and Y. Z. Lai. 1972. Thermal degradation of 1, 6-anhydro-B-D-glucopyranose. *J. Org. Chem.* 37:278-284.
28. Shafizadeh, F., S. W. Philpot, and N. Ostojic. 1971. Thermal analysis of 1, 6-anhydro-B-D-glucopyranose. *Carbohydrate Research*. 16:279-287.
29. Shulman, G. P., and P. G. Simmonds. 1968. Thermal decomposition of aromatic and heteroaromatic amino-acids. *Chem. Commun.* 17:1040-1049.
30. Simmonds, P. G. 1970. Whole microorganisms studied by pyrolysis-gas chromatography-mass spectrophotometry: significance for extra-terrestrial life detection experiments. *Appl. Microbiol.* 20:567-572.
31. Simmonds, P. G., G. P. Shulman, and C. H. Stemberge. 1969. Organic analysis by pyrolysis-gas chromatography-mass spectrometry: a candidate experiment for the biological exploration of Mars. *J. Chromatog. Sci.* 7:36-41.
32. Sneath, P. H. D. 1964. New approaches to bacterial taxonomy: use of computers. *Ann. Rev. Microbiol.* 18:335-346.

33. Sokal, R. R., and P. H. A. Sneath. 1963. Principles of numerical taxonomy. W. H. Freeman & Co. San Francisco.
34. Staub, A. M. 1965. Bacterial lipido-proteino-poly-saccharides. Extraction with trichloroacetic acid, p. 86. In Methods in carbohydrate chemistry. vol. V. Academic Press, Inc., New York.
35. Wesphal, O., O. Lüderitz, and F. Bister. 1952. "Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. 7b:148-155.